Characterisation of the microbiota of traditional fermented beverages and screening these and other populations for novel antimicrobial producers and gene clusters

A thesis presented to the National University of Ireland for the Degree of Doctor of Philosophy

By

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Table of Contents

CHAPTER 3

***In-silico* analysis highlights the frequency and diversity of type 1 lantibiotic gene clusters in genome sequenced bacteria** **94**

DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and has not been submitted for another degree, either at University College Cork or elsewhere.

Signed:_____

Alan Marsh

Date: _____

ABSTRACT

To screen for novel ribosomally synthesised antimicrobials, *in-silico* genome mining was performed on all publically available fully sequenced bacterial genomes by December, 2009. Using the nisin biosynthetic enzymes as driver sequences, 49 novel type 1 lantibiotic clusters were identified from a number of species, genera and phyla not usually associated with lantibiotic production. The prevalence of these clusters, and individual and random ORFs, indicates that these antimicrobial genes are more widespread than previously thought.

Traditional fermented beverages which have also been a source of antimicrobial producers have been developed by many different societies and are believed to confer health benefits on the consumer. Functional foods are a growing sector of the food industry, and there is a desire to develop traditional fermented beverages as functional beverages, including their application as non-dairy probiotic delivery systems, for this market. A crucial step towards commercialisation is the characterisation of the microbial content of these traditional beverages. To achieve this goal, we applied next-generation sequencing techniques to analyse the bacterial and yeast populations of the organic, symbiotically-fermented beverages kefir, water kefir and kombucha. Kefir, a milk-based beverage, is fermented by a complex association of bacteria and yeast which are contained within a polysaccharide matrix, which takes the form of kefir 'grains'. These grains and fermentates were shown to contain different bacterial populations, in that *Lactobacillus* dominate in the grain, and *Lactococcus* are the most abundant genus in the milk. Levels of *Acetobacter* varied, and *Leuconostoc* levels increased from grain to milk. The fungal component was dominated by the yeast *Kazachstania*, *Naumovozyma* and

Kluyveromyces. A number of minor components were also revealed, many of which had not previously been associated with kefir. Water kefir is similar in concept to milk kefir, in that symbiotic grains are responsible for fermentation. These grains are composed of dextran, and the medium that is fermented is a sucrose/water solution. The dominant microorganism in each of the grains and fermentates was *Zymomonas*, an ethanol-producing bacterium that had not previously been detected on such a scale. Other genera present included LAB and acetic acid bacteria. It was established that the fungal component was composed of *Dekkera*, *Hanseniaspora*, *Saccharomyces*, *Zygosaccharomyces*, *Torulaspora* and *Lachancea*. The final fermented beverage investigated, kombucha, is a fermented, tea-based beverage, with the associated microorganisms contained within a cellulosic pellicle that floats above the fermentate. The bacterial portion, long thought to be composed of *Acetobacter*, was shown to be dominated by *Gluconacetobacter*. In addition, there was a significant abundance of *Lactobacillus*. The yeast populations were found to be dominated by *Zygosaccharomyces*. Both water kefir and kombucha also contained a number of trace microorganisms which had not been discovered previously in these beverages. These studies represent the most accurate description of these populations to date, and should aid in future starter design and in determining which species are responsible for specific attributes of the beverages.

Finally, high-throughput robotics was applied to screen for the presence of antimicrobial producers associated with these beverages. This revealed a low frequency of bacteriocin production amongst the bacterial isolates, with only

lactococcins A, B and LcnN of lactococcin M being identified. However, a proteinaceous antimicrobial produced by the yeast *Dekkera bruxellensis*, isolated from kombucha, was found to be active against *Lactobacillus bulgaricus*. Partial purification determined the compound to be negatively charged, active in a pH range of 3-8 and to have a mass >30 kDa. SDS-PAGE analysis revealed the presence of 5 proteins, indicating more than one protein may be involved in antimicrobial production, and that further purification is necessary. Ultimately, the fungal component of these beverages may merit further attention with respect to the identification of antimicrobial producers.

PUBLICATIONS

This thesis generated the following publications.

Marsh, A. J., O. O'Sullivan, R. P. Ross, P. D. Cotter, and C. Hill. 2010. In silico analysis highlights the frequency and diversity of type 1 lantibiotic gene clusters in genome sequenced bacteria. BMC genomics 11.

Marsh, A. J., C. Hill, R. P. Ross, and P. D. Cotter. 2012. Strategies to Identify Modified Ribosomally Synthesized Antimicrobials. Antimicrobial Drug Discovery: Emerging Strategies 22:166-186.

Marsh, A. J., O. O'Sullivan, C. Hill, R. P. Ross, and P. D. Cotter. 2013. Sequencing-based analysis of the bacterial and fungal composition of kefir grains and milks from multiple sources. PloS one 8:e69371.

Marsh, A. J., O. O'Sullivan, C. Hill, R. P. Ross, and P. D. Cotter. 2013. Sequence-based Analysis of the Microbial Composition of Water Kefir from Multiple Sources. FEMS microbiology letters. *In press.*

Marsh, A. J., O. O. Sullivan, C. Hill, R. P. Ross, and P. D. Cotter. 2013. Sequence-based Analysis of the Bacterial and Fungal Compositions of Multiple Kombucha (Tea Fungus) Samples. Food microbiology. *In press.*

Marsh, A. J., C. Hill, R. P. Ross, and P. D. Cotter. 2013. Traditional fermented beverages with health-promoting potential: current and future perspectives. *Ready for submission.*

GLOSSARY OF TERMS

AAB	Acetic Acid Bacteria
ACE	Angiotensin 1 Converting Enzyme
Ala	Alanine
AMP	Antimicrobial Peptide
APC	Alimentary Pharmabiotic Centre
ATCC	American Type Culture Collection
Bac	Bacteriocin
BAGEL	Bacteriocin Genome Mining Tool
Bel	Belgium
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Tool
Bp	Base Pair
Ca	Canada
CFS	Cell Free Supernatant
CFU	Colony Forming Units
Da	Daltons
DGGE	Denaturing Gradient Gel Electrophoresis
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
EPS	Exopolysaccharide
F1	Forward Primer 1
Ger	Germany
GI	Gastrointestinal
Glu	Glutamic Acid
Gly	Glycine
GM17	Glucose M17 broth
H	Hour
H_2O	Water

HCl	Hydrochloric Acid
His	Histidine
HPLC	High Performance Liquid Chromatography
Ile	Isoleucine
Ire	Ireland
ITS	Internal Transcribed Spacer
kDa	Kilodaltons
L	Litre
LAB	Lactic Acid Bacteria
Lan	Lanthionine
LanB	Lanthionine Dehydratase
LanC	Lanthionine Synthetase
LB	Luria-Bertani
Leu	Leucine
Lys	Lysine
m, min	Minutes
MALDI	Matrix Laster Desorption Ionization
Met	Methionine
MEGAN	Metagenome Analyzer
MeLan	β-Methyllanthionine
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MUSCLE	Multi Sequence Comparison by Log Expectation
ug/ml	Micrograms Per Millilitre
ul	Microlitre
mg	Milligrams
mg/ml	Milligrams Per Millilitre
MRS	de Man-Rogosa-Sharpe agar
MRSA	Methicillin Resistant *Staphylococcus aureus*
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
NMR	Nuclear Magnetic Resonance

OTUs	Operational Taxanomic Units
P	P-value
PBS	Phophate Buffered Saline
PCoA	principal Co-ordinate Analysis
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
pH	Power of Hydrogen
Phe	Phenylalanine
Pro	Proline
R5	Reverse Primer 5
RNA	Ribonucleic Acid
RP	Reverse Phase
RSM	Reconstituted Skimmed Milk
rRNA	Ribosomal RNA Gene
Ser	Serine
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SRA	Sequence Read Archive
Sp	Spain
SPE	Solid Phase Extraction
TFA	Trifuoroacetic Acid
Thr	Threonine
Trp	Tryptophan
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TOMMs	Thiazole/Oxazole-Modified Microcins
Tyr	Tyrosine
U/ml	Units Per Millilitre
UK	United Kingdom
US	United States
V4-V5	Variable 16S rDNA Region

Val	Valine
WL	Wallerstein Laboratory
w/v	Weight Per Volume
YE	Yeast Extract

LIST OF FIGURES

Chapter 4

Chapter 5

Chapter 6

Chapter 7

LIST OF TABLES

Chapter 7

CHAPTER I

Strategies to identify modified ribosomally synthesized antimicrobials

Antimicrobial Drug Discovery: Emerging Strategies 2012 CABI Publishing

Eds: Tegos, G. and Mylonakis, E.

1.1 ABSTRACT

As a consequence of their frequently high potency, their gene-encoded nature and the rarity with which resistance occurs, post-translationally modified, ribosomally synthesized, antimicrobial peptides are viewed as having considerable potential as novel chemotherapeutic agents. Perhaps the best studied of these modified antimicrobial peptides are the lantibiotics which are distinguished by the fact they contain the unusual, modified amino acids, lanthionine and β-methyllanthionine. Traditional approaches to lantibiotic discovery have involved the use of well-diffusion, deferred antagonism as well as broth/photometric-based assays. However, these have been supplemented with strategies that involve the use of high-throughput technologies, PCR-based detection, engineering of known lantibiotics and *in-silico* mining. Here we review the strategies that have been successfully employed to identify novel lantibiotics and other modified, ribosomally synthesized antimicrobial peptides.

1.2 Introduction

This chapter describes the different strategies that can be, and have been, employed to identify/create novel post-translationally modified, ribosomally-synthesized antimicrobial peptides. These are peptides which are first synthesized as immature peptides which then undergo enzyme-mediated, post-translational modification and the cleavage of a N-terminal leader region to form fully-functional, mature peptides. In recent years the number of such antimicrobials has increased noticeably through the identification of novel forms of well-known families, such as the lantibiotics, and the inclusion of additional families of antimicrobials, such as the cyanobactins, thiopeptides, microviridins and amatoxins. Some of these are thought to be viable alternatives to the antibiotics which are currently employed clinically and, indeed, it is hoped that their use could stave off the issues arising as a consequence of resistance to existing antimicrobials. Here we will review the strategies employed to identify a representative, and possibly the most extensively studied, family of modified antimicrobial peptides, the lantibiotics, and highlight how these have been and can be applied to screen for other modified peptides.

1.3 The lantibiotics

Bacteriocins are small, heat-stable antimicrobial peptides, produced by bacteria, and are typically active against species closely related to the producer, but can also exhibit activity across genera. Bacteriocin producers are naturally immune to their own bacteriocins as a consequence of possessing specific self-protective

mechanisms. There are a wide range of existing and potential commercial and medicinal applications for these peptides. Due to the continuous discovery of novel antimicrobial peptides, one of the original bacteriocin, and highly cited, classification systems, devised by Klaenhammer (Klaenhammer, 1993) to classify bacteriocins produced by Gram positive, lactic acid, bacteria, has undergone several revisions. The most recent classification (Rea et al., 2011) represents an updating of a system proposed by Cotter et al (Cotter et al., 2005b) and recommends that Gram positive bacteriocins, and indeed bacteriocins in general, could be divided into 2 Classes, consisting of Class I, the post-translationally modified bacteriocins, and Class II, the unmodified bacteriocins. Class I Gram positive bacteriocins can be subdivided into 3 groups, (a) the lantibiotics/lantipeptides, (b) the labrinthopeptides and (c) the sactibiotics, although further groups are likely to be ultimately added. These can be further subdivided according to amino acid composition and the number of peptides involved. Gram positive bacteriocins from within Class II can be divided into 4 groups, which can also be further subdivided (Rea et al., 2011) (**Figure 1a**).

The lantibiotics/lantipeptides, i.e. Class Ia bacteriocins, which are the primary focus of this manuscript contains 4 subgroups. Types 1 and 2 are the lantibiotics (so named because they are lanthionine-containing antibiotics) while types 3 and 4 are the bacteriocin-like lantipeptides. The generic name given to lantibiotic structural prepropeptides is LanA. These are gene-encoded (by *lanA*) and undergo subsequent modification via cleavage of a N-terminal leader region (required for export and directing post-translational modification)

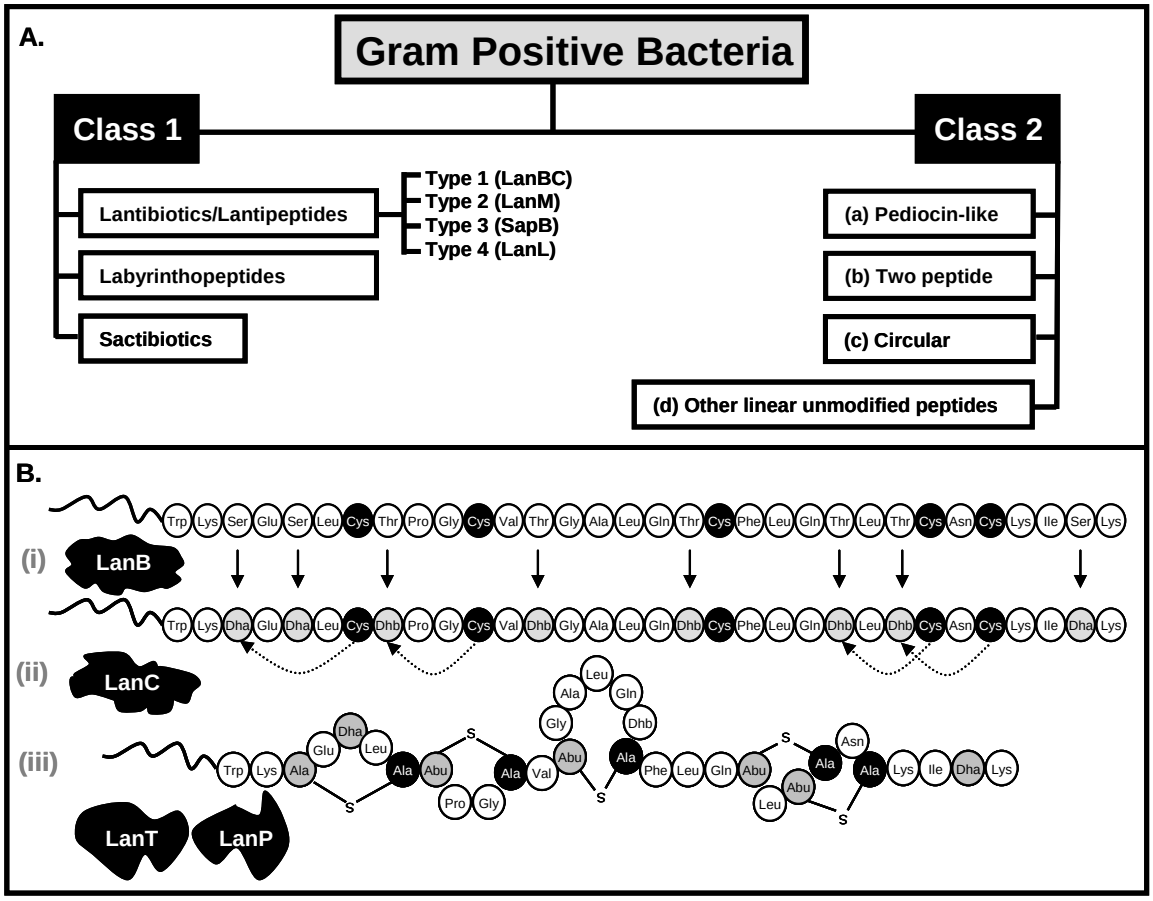

Figure 1

A. The Classification of Gram-positive bacteriocins

B. The enzyme-mediated synthesis of the lantibiotic subtilin

(i) Lanthionine dehydratase (LanB) catalyses the dehydration of Serine and Threonine residues to form Dha and Dhb respectively

(ii) Lanthionine synthetase (LanC) catalyses a condensation reaction between the sulphydryl group of cysteines and the dehydrated residues

(iii) Following the cleavage of the leader peptide by LanP, LanT transports the mature peptide across the cell membrane

and the modification of the C-terminal (propeptide) region. The modifications which are specifically associated with this family of peptides occur as follows; specific serine and threonine residues contained within the propeptide are dehydrated to form dehydroalanine and dehydrobutyrine, respectively. When these modified residues interact with an intrapeptide cysteine, a thioether bond is formed resulting in the formation of the eponmymous amino acids, lanthionine (Lan, from Dha) or β-methyl lanthionine (meLan, from Dhb), respectively (**Figure 1b**). These structures play a critical role in the antimicrobial activity of the peptides such as, in the case of the prototypical type 1 lantibiotic, nisin, facilitating the binding to a lipid II target (Hsu et al., 2004).

Similarly, the contribution of these structures to the resistance of lantibiotics to high temperature and proteolytic enzymes is also apparent (Suda et al., 2010). The distinction between type 1 and type 2 lantibiotics is based on the modification enzymes involved. Type 1 peptides are modified by two catalytic enzymes i.e. LanB, a lanthioine dehydratase, and LanC, a lanthionine synthetase. Type 2 lantibiotics are modified by LanM enzymes which perform both the dehydratase and cyclase functions. The *lanM/lanBC* genes are the most highly conserved genes within lantibiotic gene clusters; a trait which has been utilized, through the use of degenerate primers and *in-silico* screens to identify novel lantibiotic-encoding clusters (see below). The type 1 and 2 lantibiotics can be further subdivided on the basis of homology with respect to the amino acid sequence of the prepropeptide. There also exist a number of two-peptide lantibiotics i.e. lantibiotics which are active through the combined activity of two lanthionine-containing peptides (Lawton et al., 2007b).

The aforementioned nisin is currently the best studied of all bacteriocins and has been used as a food preservative in over 50 countries. However, as a consequence of the emergence of microbial resistance to therapeutic antibiotics, there have been a number of investigations into the use of nisin and novel lantibiotics against clinically relevant pathogens. This stems from the fact that the antimicrobial activity of lantibiotics can be greater than that of classical antibiotics and the fact that they can target very sensitive components of the bacterial cell, such as the aforementioned lipid II. To date, approximately 60 lantibiotics have been isolated from Gram positive bacteria (Firmicutes and Actinobacteria) (**Table 1**). Although the majority of these have been identified using traditional bioactivity-based screens, we are at the dawn of a new age in technological advancements, in which *in-silico*, molecular and bioengineering-based approaches can complement and, potentially ultimately supersede, the reliable but increasingly out-dated, culture-based methods employed for lantibiotic discovery. Here we review the use of these various approaches (**Figure 2**).

Table 1. The screening method and source employed to discover known lantibiotics

Lantibiotic	Producer	Class/Group	Mode of Discovery	Strain Source	Reference
Nisin A	*Lactococcus lactis*	Type 1, Nisin Group	Incidental (Observed Inhibition)	Dairy Culture	(Whitehead 1933)
Nisin Z	*Lactococcus lactis*	Type 1, Nisin Group	Not Specified	Not Specified	(Mulders et al., 1991)
Nisin F	*Lactococcus lactis*	Type 1, Nisin Group	Overlay Assay	Fresh Water Catfish Faeces	(de Kwaadsteniet et al., 2008)
Nisin U	*Streptococcus uberis*	Type 1, Nisin Group	Deferred Antagonism Assay	Not Specified	(Wirawan et al., 2006)
Nisin U2	*Streptococcus uberis*	Type 1, Nisin Group	Deferred Antagonism Assay	Not Specified	(Wirawan et al., 2006)
Nisin Q	*Lactococcus lactis*	Type 1, Nisin Group	Not Specified	Hikosan River Water	(Zendo et al., 2003)
Lacticin NK34	*Lactococcus lactis*	Type 1, Nisin Group	Lawn Spotting	Korean Fermented Fish (Jeotgal)	(Lee et al., 2008)
Subtilin	*Bacillus subtilis*	Type 1, Nisin Group	Incidental (Observed Inhibition)	Not Specified	(Jansen and Hirschmann, 1944)
Ericin (A and S)	*Bacillus subtilis*	Type 1, Nisin Group	Not Specified	Not Specified	(Stein et al., 2002)
Entianin	*Bacillus subtilis*	Type 1, Nisin Group	Microtiter Autoinduction Bioassay	Tunisian Desert	(Fuchs et al., 2011)
Microbisporicin	*Microbispora corallina*	Type 1, Nisin Group	High-throughput Screen	Uncommon Environmental Actinomycetes	(Castiglione et al., 2008)
Planosporicin	*Planomonospora alba*	Type 1, Nisin Group	High-Throughput Screen	Uncommon Environmental Actinomycetes	(Castiglione et al., 2007)
Clausin	*Bacillus clausii*	Type 1, Epidermin Group	Not Specified	Not Specified	(Bouhss et al., 2009)
Staphylococcin T	*Staphylococcus cohnii*	Type 1, Epidermin Group	Not Specified	Human Throat	(Furmanek et al., 1999)
Epidermin	*Staphylococcus epidermidis*	Type 1, Epidermin group	Not Specified	Not Specified	(Allgaier et al., 1986)
Gallidermin	*Staphylococcus gallinarum*	Type 1, Epidermin group	Not Specified	Chicken Crests	(Kellner et al., 1988)
Mutacin I	*Streptococcus mutans*	Type 1, Epidermin group	Stab Assay	Several (Streptococcus Mutans)	(Hamada and Ooshima, 1975)
Mutacin III	*Streptococcus mutans*	Type 1, Epidermin group	Not Specified	Caries-Active Female	(Qi et al., 1999)

Table 1. The screening method and source employed to discover known lantibiotics

Lantibiotic	Producer	Class/Group	Mode of Discovery	Strain Source	Reference
Mutacin 1140	*Streptococcus mutans*	Type 1, Epidermin Group	Deferred Antagonism Assay	Not Specified	(Hillman et al., 1998)
Mutacin B-Ny266	*Streptococcus mutans*	Type 1, Epidermin group	Deferred Antagonism Assay	Not Specified	(Morency et al., 1995)
Staphylococcin AU-26	*Staphylococcus aureus*	Type 1, Epidermin Group	Deferred Antagonism Assay	Vagina	(Scott et al., 1992)
BSA	*Staphylococcus aureus*	Type 1, Epidermin Group	*In-Silico*	CA-MRSA Isolates	(Daly et al., 2010)
Streptin	*Streptococcus pyogenes*	Type 1, Streptin group	Molecular	Not Specified	(Karaya et al., 2001)
Pep5	*Staphlyococcus epidemidis*	Type 1, Pep5 Group	Deferred Antagonism Assay	Not Specified	(Kellner et al., 1989)
Epicidin 280	*Staphlyococcus epidemidis*	Type 1, Pep5 Group	Not Specified	Not Specified	(Heidrich et al., 1998)
Epilancin K7	*Staphlyococcus epidemidis*	Type 1, Pep5 Group	Not Specified	Human Nasal Cavity	(Pulverer and Jeljaszewicz, 1975)
Epilancin 15X	*Staphlyococcus epidemidis*	Type 1, Pep5 Group	Not Specified	Wound Infection	(Ekkelenkamp et al., 2005)
Salivaricin 9	*Streptococcus salivarius*	Type 2, Lacticin 481 Group	*In-Silico*	Not Specified	(Wescombe et al., 2011)
Lacticin 481	*Lactococcus lactis*	Type 2, Lacticin 481 Group	Spot Assay	Dairy	(Picard et al., 1990)
Thermophilin 1277	*Streptococcus thermophilus*	Type 2, Lacticin 481 Group	Not Specified	Raw Milk	(Kabuki et al., 2007)
Bovicin Hj50	*Streptococcus bovis*	Type 2, Lacticin 481 Group	Agar Well Diffusion Assay	Raw Milk	(Xiao et al., 2004)
Macedocin	*Streptococcus macedonicus*	Type 2, Lacticin 481 Group	Well Diffusion/Overlay Assays	Greek Kasseri Cheese	(Georgalaki et al., 2000)
Butyrivibriocin AR10	*Butyrivibrio fibrisolvens*	Type 2, Lacticin 481 Group	Deferred Antagonism Assay	Rumen	(Kalmokoff and Teather, 1997)
Ruminococcin A (RumA/B)	*Ruminococcus gnavus*	Type 2, Lacticin 481 Group	Not Specified	Male Gut Flora	(Ramare et al., 1993)
Variacin	*Micrococcus varians*	Type 2, Lacticin 481 Group	Well Diffusion Method	Salami	(Pridmore et al., 1996)

Table 1. The screening method and source employed to discover known lantibiotics

Lantibiotic	Producer	Class/Group	Mode of Discovery	Strain Source	Reference
Streptococcin A-FF22	*Streptococcus pyogenes*	Type 2, Lacticin 481 Group	Deferred and Simultaneous Antagonism Assays	Patient Throat Cultures	(Tagg et al., 1973)
Butyrivibriocin OR79A/OR79B	*Butyrivibrio fibrisolvens*	Type 2, Lacticin 481 Group	Deferred Antagonism Assay	Dairy Cow Rumen	(Kalmokoff et al., 1999)
Mutacin II	*Streptococcus mutans*	Type 2, Lacticin 481 Group	Deferred Antagonism Assay	Saliva of Healthy Children	(Novak et al., 1994)
Mutacin K8 (mukA123+A')	*Streptococcus mutans*	Type 2, Lacticin 481 Group	Deferred Antagonism Assay	Not Specified	(Robson et al., 2007)
Salivaricin A1	*Streptococcus pyogenes*	Type 2, Lacticin 481 Group	Molecular (Hybridization)	Oral Strain	(Simpson et al., 1995)
Salivaricin A	*Streptococcus pyogenes*	Type 2, Lacticin 481 Group	Deferred Antagonism Assay	Oral Strain	(Ross et al., 1993)
Salivaricin A2	*Streptococcus salivarius*	Type 2, Lacticin 481 Group	Deferred Antagonism Assay	Human Saliva	(Hyink et al., 2007)
Salivaricin B	*Streptococcus salivarius*	Type 2, Lacticin 481 Group	Deferred Antagonism Assay	Human Saliva	(Hyink et al., 2007)
Salivaricin A2/3/4/5	*Streptococcus rattus*	Type 2, Lacticin 481 Group	Deferred Antagonism Assay	Human Saliva	(Wescombe et al., 2006)
Nukacin ISK-1	*Staphylococcus warneri*	Type 2, Lacticin 481 Group	Not Specified	Nukadoko, Fermented Rice Bran	(Kimura et al., 1998)
Nukacin 3299	*Staphylococcus simulans*	Type 2, Lacticin 481 Group	Deferred Antagonism Assay	Bovine Mastitis Cases	(Nascimento et al., 2002)
Nukacin KQU-131	*Staphylococcus hominis*	Type 2, Lacticin 481 Group	Not Specified	Thai Fermented Marine Fish	(Wilaipun et al., 2008)
Mersacidin	*Bacillus sp*	Type 2, Mersacidin Group	Not Specified	Indian Soil Sample	(Chatterjee et al., 1992)
Plantaricin-C	*Lactobacillus plantarum*	Type 2, Mersacidin Group	Deferred Antagonism/Well Diffusion Assay	Fermentations (Without Starter Cultures)	(Gonzalez et al., 1994)
Michiganin A	*Clavibacter michiganensis*	Type 2, Mersacidin Group	Not Specified	Plant Pathogen	(Holtsmark et al., 2006)
Actagardine	*Actinoplanes liguriae*	Type 2, Mersacidin Group	Not Specified	Indian Garden Soil	(Parenti et al., 1976)
Ala (0) - Actagardine	*Actinoplanes liguriae*	Type 2, Mersacidin Group	Not Specified	Not Specified	(Vertesy et al., 1999)

Table 1. The screening method and source employed to discover known lantibiotics

Lantibiotic	Producer	Class/Group	Mode of Discovery	Strain Source	Reference
Deoxyactagardine B (DAB)	*Actinoplanes liguriae*	Type 2, Mersacidin Group	Molecular (Hybridization)	Not Specified	(Boakes et al., 2010)
Smb (SmbA/B)	*Streptococcus mutans*	Type 2, LtnA2/Mersacidin	Molecular	Oral Strain	(Yonezawa and Kuramitsu, 2005)
Plantaricin W (Plwa/b)	*Lactobacillus plantarum*	Type 2, Mersacidin/LtnA2	Not Specified	Wine (Pino Noir)	(Holo et al., 2001)
Staphylococcin C55 (C55α/β)	*Staphylococcus aureus*	Type 2, Mersacidin/LtnA2	Lawn Spotting/CFU Counts	Superficial Skin Lesions	(Dajani and Wannamaker, 1969)
Haloduracin (BhaA1/A2)	*Bacillus halodurans*	Type 2, Ltn α	*In-Silico*	Soil	(Lawton et al., 2007a) (Mclerren et al., 2006)
Lichenicidin (BliA1/A2)	*Bacillus licheniformis*	Type 2, Ltn α	*In-Silico*	Not Specified	(Begley et al., 2009)
Lacticin 3147 (Ltnα/β)	*Lactococcus lactis*	Type 2, Mersacidin/Ltn α	Incidental (Observed Inhibition)	Cheese Culture	(Ryan et al., 1995)
Cytolysin Ll/Ls	*Bacillus halodurans*	Type 2, Cytolysin Group	Deferred Antagonism Assay	Not Specified	(Brock and Davie)
Lactocin-S	*Lactobacillus sakei*	Tyoe 2, Lactocin S Group	Deferred Antagonism Assay	Fermented Dry Sausage	(Mortvedt and Nes, 1990)
Cinnamycin	*Streptoverticillum griseoverticillatum*	Type 2, Cinnamycin Group	Not Specified	Japenese Soil	(Benedict et al., 1952)
Duramycin A	*Streptomyces cinnamomeus*	Type 2, Cinnamycin Group	Not Specified	Not Specified	(Shotwell et al., 1958)
Duramycin B	*Streptomyces griseoverticillatus*	Type 2, Cinnamycin Group	Phospholopase A Inhibitor Screen	Not Specified	(Fredenhagen et al., 1990)
Duramycin C	*Streptomyces griseoluteus*	Type 2, Cinnamycin Group	Phospholopase A Inhibitor Screen	Not Specified	(Fredenhagen et al., 1990)
Ancovenin	*Streptomyces sp.*	Type 2, Cinnamycin Group	Enzyme Inhibitor Screen	Tokyo Soil Sample	(Kido et al., 1983)
Carnocin UI49	*Carnobacterium sp.*	Not Determined	Spot Assay	Fresh Fish	(Stoffels et al., 1992)
Paenibacillin	*Paenibacillus polymyxa*	Not Determined	Deferred Antagonism Assay (Filter Membranes)	Fermented Foods	(He et al., 2007)

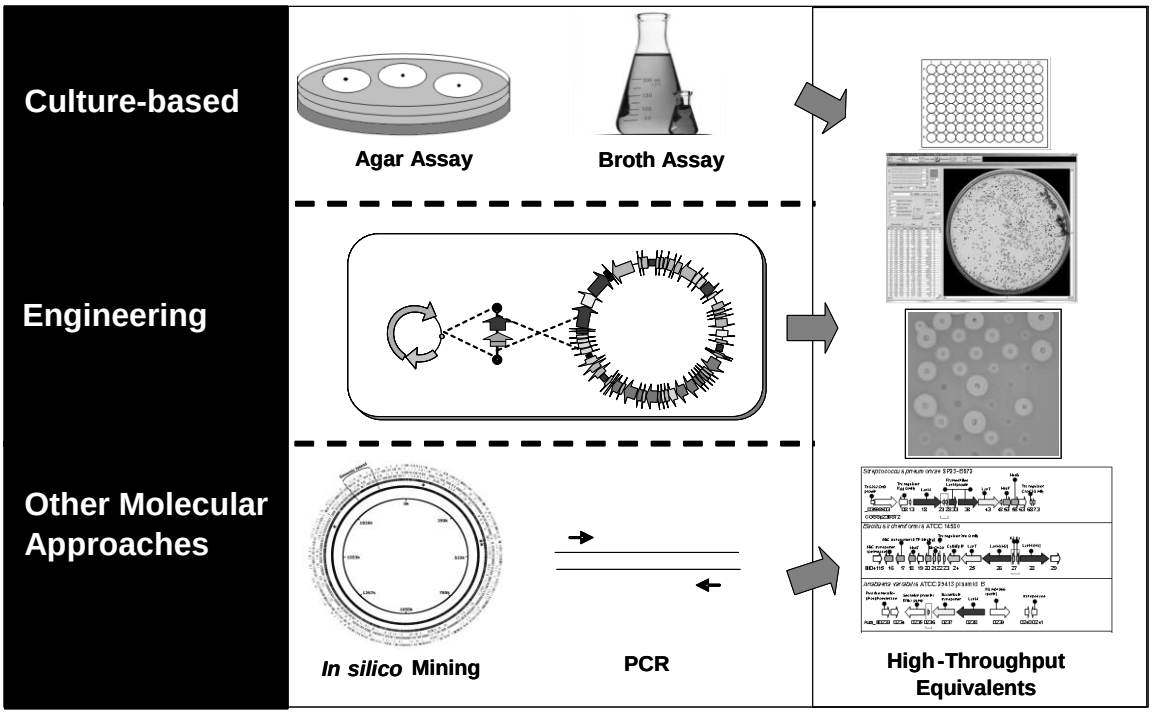

Figure 2

Methods used to screen for novel peptides. These include traditional culture-based analyses such as well diffusion, deferred antagonism and broth assays, bioengineering and other molecular approaches such as PCR, mutagenesis and in-silico mining.

1.4 Traditional (culture-based) screening methods

The first historical report of bacteriocin production dates to 1877 when Pasteur and Joubert (Pasteur and Joubert, 1877) noticed that bacteria isolated from urine samples inhibited *Bacillus anthracis*. This was followed in 1925 by a report prompted by the observation that species of *Escherichia coli* inhibited the growth of one another. A 1928 study of the limiting factors in lactic fermentation concluded that cell inhibition was "determined by the concentration of a definite, soluble and diffusible substance excreted by the cells". Similarly, in 1933, Whitehead, by process of elimination, deduced that the inability of a certain milk sample to accommodate the growth of starter lactic acid bacteria was due to a proteinaceous, heat stable, inhibitory substance produced by two strains of streptococci (since re-classified as lactococci) found in the original milk sample (Whitehead, 1933). This substance was later named nisin (Mattick and Hirsch, 1947).

Despite the technological revolution which has occurred in the intervening years, the majority of lantibiotics have been identified using methods that are not extensively dissimilar from those employed in the early years of lantibiotic research and which rely on identifying the ability of one bacterial strain (producer) to inhibit the growth of another (indicator). However, rather than its identification as a consequence of the coincidental observation of antimicrobial activity, as was the case for nisin, the identification of novel antimicrobials using this strategy has most frequently been as a result of purpose-built screens employing specific techniques and numerous bacterial species. These can be performed in a variety of ways. One of the most common

of these traditional approaches is the deferred antagonism assay. This involves pipetting a set volume of the producer strain onto the appropriate agar, and following incubation, semi-molten agar seeded with an indicator organism is then overlaid. An alternative is the agar well diffusion assay. In this case, the appropriate molten agar is inoculated with indicator cells. This is allowed to cool before wells are bored. To each well, cell-free supernatant from the producer microorganism or purified peptide is added and, after appropriate incubation (to allow growth of the indicator and diffusion of the antimicrobial), antimicrobial activity can be assessed.

Broth/photometric-based assays can also be employed. Here, the indicator strains are inoculated into broth and cell free supernatant from the producer organism or purified peptide is added prior to incubation. After a period of time, cell density is measured using, for example, an absorbance plate reader, revealing if the growth of the indicator culture has been inhibited. With the exception of standardized assays, such as those recommended by the National Committee for Clinical Laboratory Standards for minimum inhibitory concentration determination (Marshall et al., 1996), the specific details as to how agar and broth-based assays are carried out can vary from laboratory to laboratory. Regardless of these variations, a significant limitation of these functional assays is that novel lantibiotic producers may be overlooked if the parameters employed, such as pH, incubation temperature, time of incubation, carbohydrate source and indicator strain selection, are not optimal. Once antimicrobial activity is detected and revealed, through the use of proteases such as proteinase K, pepsin, trypsin and α-chymotrypsin, to be proteinaceous

in nature, the next step most frequently involves efforts to purify the peptide, most frequently via high-performance liquid chromatography (HPLC) coupled with mass spectrometry. The family of antimicrobials to which the inhibitor belongs can be definitively established through nuclear magnetic resonance (NMR) spectroscopy, although other efforts to elucidate the amino acid sequence of the peptide, through N-terminal peptide sequencing or mass spectrometry, can be valuable. The availability of data with respect to the amino acid sequence (or partial amino acid sequence) of the peptide can in turn also enable the identification of the gene cluster of interest through reverse genetics or upon genome sequencing of the strain.

Culture-based approaches have facilitated the identification of novel lantibiotic producers from a variety of sources such as milk, fermented foods, oral cavities, faecal samples and soil. The frequent use of a starting material that possesses a diverse microbiota stems from the supposition that microbes that need to compete in such environments are most likely to be antimicrobial producers. While here we provide just a few, select, examples, the source of other lantibiotic-producing bacteria is collated in **Table 1**. Ryan *et al*. (Ryan et al., 1996) chose to screen lactococci isolated from kefir grains with the intention of finding a *Lactococcus* capable of producing a potent bacteriocin. It was anticipated that such a strain could be used commercially as a starter strain that more successfully control spoilage and pathogenic microbes in food fermentations. It was hypothesised that kefir grains could be a rich source of bacteriocin-producing lactococci in that, while kefir grains contain a rich microbiota that is chiefly composed of yeast and lactobacilli, lactococci

predominate in kefir-fermented milk. Ultimately, a number of antimicrobial-producing lactococci were isolated, including strain DPC 3147 which was ultimately found to be the producer of a two-peptide lantibiotic, named lacticin 3147. This lantibiotic has since become one of the most extensively studied lantibiotics (Carroll et al., 2010, Cotter et al., 2006, Draper et al., 2009, Gardiner et al., 2007). The discovery of the lantibiotic butyrivibriocin AR10 stemmed from the study of bacteria isolated from the rumen of cattle (Kalmokoff and Teather, 1997). The rumen is the primary site of microbial fermentation of ingested feed in cattle, and both the rumen and ruminal fluid are known to have an inhibitory effect on nonruminal bacteria. 49 isolates of *Butyrivibrio fibrisolvens*, and a single isolate of *Butyrivibrio crossotus*, were isolated from the rumen and their ability to produce antimicrobials was assessed using a deferred antagonism assay, using other *Butyrivibrio* as indicators. 25 isolates were shown to produce inhibitory agents, of which 18 were sensitive to protease digestion. The antimicrobial produced by one such isolate was chosen for purification and further analysis which culminated in the identification of butyrivibriocin AR10, the first ruminal anaerobe-associated bacteriocin (Kalmokoff and Teather, 1997).

Humans can also host many lantibiotic producers. Indeed, staphylococcin Au-26 was characterized from a vaginal isolate of *Staphylococcus aureus* after a study was initiated following the observation that vaginal *S. aureus* associated with toxic-shock syndrome (TSS) were producers of bacteriocins (Scott et al., 1992). It was postulated that this antimicrobial may confer a competitive advantage to the infectious bacteria over the indigenous

flora of lactobacilli and so a deferred antagonism test procedure was carried out using endocervical lactobacilli as indicators, which resulted in the isolation of *S. aureus* strain 26 and the associated lantibiotic, staphylococcin Au26 (Scott et al., 1992). Interestingly, the same lantibiotic was also associated with many community-associated methicillin-resistant *S. aureus* (CA-MRSA) isolates (Daly et al., 2010). Finally, soil has also proven to be a rich repository for producers of lantibiotics and it is from this source that ancovenin, mersacidin and actagardine (Chatterjee et al., 1992, Kido et al., 1983, Parenti et al., 1976) were identified. Ancovenin was discovered while screening 5,200 samples for microbial enzyme inhibitors. Ancovenin was purified from the culture broth of *Streptomyces* sp. No. A647P-2, a strain isolated from a soil sample collected in Tokyo and was initially indentified on the basis of its specific inhibitory action against angiotensin I converting enzyme (ACE) rather than as a consequence of possessing antimicrobial activity (Kido et al., 1983).

More recently, efforts have been made to scale up the processes involved in culture-based screening for antimicrobials. The lantibiotics planosporicin and microbisporicin were found as a direct result of a biological activity-guided high-throughput screening (HTS)-based strategy designed to target novel peptidoglycan biosynthesis inhibitors (Castiglione et al., 2007, Castiglione et al., 2008). This HTS approach relied on the use of robotics to investigate 40,000 actinomycetes isolated from the environment, which were then fermented to yield a library of 120,000 broth extracts. The initial step of the antimicrobial activity assay was to assess the ability of the extracts to inhibit the growth of *S. aureus* (in its cell wall-deficient state) in liquid microplate

assays. The next stage was to disregard those extracts likely to be antimicrobials that had already been characterised i.e. β-lactam antibiotics and glycopeptides. These were eliminated through assays with a β-lactamase cocktail or through use of a D-Ala-D-Ala affinity resin, respectively. This resulted in the identification of 5 novel lantibiotics, including the aforementioned planosporicin and microbisporicin (Castiglione et al., 2007, Castiglione et al., 2008). Notably, microbisporicin was subsequently found to have a wide antimicrobial spectrum, being active against many of the Gram-positive species of medical significance and against some Gram negative pathogens (Castiglione et al., 2008), making it one of the most potent lantibiotics identified to date.

1.5 Molecular screening methods

The second half of the 20th century saw the advent of molecular genetics, which had revolutionised many biological fields. Its relevance to the identification and creation of novel peptides lies in its ability to detect and manipulate genes and, consequently, gene products. Polymerase Chain Reaction (PCR) coupled with gene sequencing have been particularly valuable tools with respect to the detection of novel lantibiotic-encoding operons and will be discussed here. The manipulation of genes using PCR-mediated approaches is also relevant and will be discussed later in this review.

A representative example of the use of PCR to identify a novel lantibiotic-encoding cluster was provided during the recent discovery of the type 2 lantibiotic Salivaricin 9 (Wescombe et al., 2011). During the course of previous studies it had been established that *Streptocccus salivarius* strain 9

produces the lantibiotic SalA4 (Wescombe et al., 2006). However, it was soon realized that this was not the only antimicrobial produced by the strain. Using degenerate primers designed to bind to and amplify regions conserved across all *lanM*s, the *lanM* associated with Salivaricin 9 biosynthesis was found to be present on the genome of *S. salivarius* strain 9. Inverse PCR was then used to amplify and sequence the region around *lanM*. Percentage similarities to previously sequenced genes/gene products were determined using Basic Local Alignment Search Tool (BLAST), an alignment program which compares base-pair similarities of sequences against sequence databases. In this way, the sequenced 6277 bp region was shown to contain genes characteristic of a lantibiotic operon, including a structural gene and regulatory elements. It should be noted, however, that subsequent culture-based approaches were required to confirm that a novel antimicrobial was indeed produced (Wescombe et al., 2011) and to determine its antimicrobial spectrum.

Similar, degenerate primer-based approaches have been employed to facilitate the identification of the gene clusters associated with the production of Smb (Yonezawa and Kuramitsu, 2005) and BHT-A (Hyink et al., 2005). It is also noteworthy that an alternative set of degenerate *lanM* primers has recently been designed to reflect the availability of an even larger collection of *lanM* sequences (O'Sullivan et al., 2011). A corresponding approach, using *lanB* and *lanC* degenerate primers pairs, led to the identification of the gene cluster associated with the type 1 lantibiotic, Nisin U (Wirawan et al., 2006). The degenerate primer pairs were designed on the basis of conserved amino acid sequences within the LanBs of streptin, pep5, nisin, epidermin, epicidin, and

subtilin, while the *lanC* primers were constructed on the basis of conserved regions within the corresponding LanC proteins as well as that associated with salivaricin A production (Wirawan et al., 2006). Despite the success of these approaches they have not, to date, been employed as part of high-throughput PCR-based approaches to identify novel lantibiotic-associated clusters from collections of strains.

A quite different molecular tool has been employed in the past to identify novel producers of the lantibiotic nisin from human milk (Beasley and Saris, 2004). This took advantage of the fact that, in addition to being an antimicrobial peptide, nisin is also an auto-inducer of its own production (Chandrapati and O'Sullivan, 1999). The milk was initially screened using an agar diffusion test, where milk was spotted onto Luria-Bertani (LB) agar, and overlaid with a *Micrococcus luteus* indicator. 20 colonies producing zones of inhibition were selected and their identity was determined by partial 16S rRNA sequencing. It was found that the strains isolated were representatives of *L. lactis* subsp. *lactis* and, as a consequence of the identity of the microbes, it was suspected that they might be producers of nisin. However, characterisation of the strains revealed them to be quite different from nisin-producing strains previously isolated from cows' milk. To establish that the antimicrobial activity observed was indeed due to nisin production, a microplate assay designed to detect nisin, on the basis of the fusion of a nisin-inducible promoter to a gene encoding a green fluorescent protein reporter (Reunanen and Saris, 2003), was used. Ultimately, these investigations indicated that approximately 30% of human breast milk contains nisin-producing bacteria.

1.6 Bioinformatic approaches

As a consequence of the advent of next-generation sequencing technologies, the number of bacterial genome sequences available has increased dramatically. Many of these genome sequences are freely available and accessible via online databases and can be mined for particular genes, including bacteriocin-encoding gene clusters, and their predicted products. The benefits from this approach with respect to lantibiotic discovery have been highlighted on a number of occasions in recent years. Indeed, although approximately 60 lantibiotics have been discovered to date, this number is greatly enhanced when *in-silico* identified lantibiotics are included in this estimate.

Some of the first studies to use a bioinformatic approach to identify novel bacteriocin-associated genes led to the identification of a gene cluster encoding the two-peptide class 2 lantibiotic, haloduracin, within the genome of *Bacillus halodurans* C-125. These genes were identified on the basis of the homology between the predicted prepropeptides encoded and those of the prototypical two peptide lantibiotic, lacticin 3147, and the related one peptide lantibiotic, mersacidin (McClerren et al., 2006, Twomey et al., 2002). Analysis of the remainder of the gene cluster revealed the presence of two *lanM*s, a *lanT* (transporter-encodin gene) and two sets of *lanEFG* genes (encoding ABC transporters potentially involved in immunity). The lantibiotic encoded by these genes was accessed through the *in vitro* reconstitution of lantibiotic synthesis (McClerren et al., 2006) and through studies with the C-125 strain and associated cell-free supernatant (Lawton et al., 2007a). This approach has since been used on an even larger scale to reveal additional type 2 gene clusters

(Begley et al., 2009). In this case computational analyses was carried out to search bacterial genome sequences for genes potentially encoding homologs of the lacticin 3147 modification enzyme, LtnM1. This resulted in the generation of a list of 89 relevant genes. Notably, 61 of these were predicted to be produced by strains not previously thought to be lantibiotic producers and 5 representatives were selected for detailed bioinformatic analysis. One associated strain, *Bacillus licheniformis* ATCC 14580, was selected for wet-lab investigations which led to the discovery of lichenicidin, a two-peptide lantibiotic which exhibits antimicrobial activity against *Listeria monocytogenes*, MRSA and vancomycin-resistant enterococcus.

Inspired by the discovery of lichenicidin, a more recent *in-silico* search was undertaken to identify additional novel LanM homologs in DNA databases (O'Sullivan et al., 2011). LtnM1 was again used as a driver sequence to mine publically available microbial genomes and by this time the number of LanM-encoding genes had increased to 124. In this instance 9 genes, and associated clusters, were subjected to an in-depth bioinformatic analysis. In addition, the metagenomic portal CAMERA (Seshadri et al., 2007) was used to search for LtnM1 homologs amongst all publicly available metagenomic datasets, which revealed a further 11 *lanMs* associated with lantibiotic gene clusters from a number of diverse environments (O'Sullivan et al., 2011).

A corresponding study has utilized the nisin modification enzymes, NisB and NisC, as driver sequences to identify novel type 1 lantibiotics (Marsh et al., 2010). In total, 49 previously unrecognised lantibiotics were uncovered in the genomes of microbes isolated from a variety of environments, such as deep sea

hydrothermal vents, the soil, the gastrointestinal tract and skin surfaces. Notably, the microbes in question included those from phyla (*Bacteroidetes* and *Chylamydiae*) not previously associated with the production of lantibiotics. The availability of this data again facilitated the identification of common motifs and residues while also permitting phylogenetic analysis and the construction of evolutionary trees which highlighted phylogenetic relatedness and diversity (Marsh et al., 2010).

The mounting interest in the discovery of new bacteriocins utilizing the ever-expanding database of genomic information is also reflected by the development of the web-based bacteriocin genome mining tool, namely BAGEL (de Jong et al., 2006) and the updated BAGEL 2 (de Jong et al., 2010). BAGEL can identify novel bacteriocin clusters using knowledge-based bacteriocin databases and motif databases and also analyses the sequence surrounding the gene of interest for bacteriocin-associated proteins (transporters, immunity genes etc). Importantly, open reading frame detection acts independently of existing annotations and therefore can detect small structural peptides that may otherwise be overlooked. A theoretical drawback is that reliance on motifs will only uncover bacteriocins sharing homology with those already described. It should be noted that the association between gene clusters identified *in-silico* and lantibiotic production is putative until such time as antimicrobial activity is confirmed through analysis of the strain in question or heterologous expression of the relevant genes in an appropriate host (Majchrzykiewicz et al., 2010). However, the studies that have taken place to date indicate the usefulness of these approaches.

It is also notable that the popularity of bacteriocins is such that a database has been generated which is dedicated to the organization of bacteriocin-related data from the literature. This database, known as BACTIBASE, contains information relating to these bacteriocins, including calculated or predicted structural and physiochemical properties of bacteriocins produced by Gram positive and Gram negative bacteria (Hammami et al., 2010).

1.7 Bioengineering of lantibiotics

An alternative approach to the identification of novel lantibiotics involves the generation of peptides with enhanced antimicrobial activities. The fact that bacteriocins are gene-encoded facilitates the use of bioengineering to generate novel derivatives which are, in essence, novel bacteriocins. This contrasts with the situation with the majority of 'classical' antibiotics which are non-ribosomal and are synthesized by multi-enzyme complexes in the absence of a specific structural precursor, thus making genetic manipulation more challenging.

The tolerance of lantibiotics to change is evident from nature in that natural variants of lantibiotics can exist. Nisin is a prime example in that the nisin family includes nisin A, nisin Z, nisin Q, nisin F, nisin U and nisin U2, although in the latter two cases the peptides differ more substantially from nisin A and their description as nisin variants is debatable (Piper et al., 2010). Over the past 20 years there have been several efforts to harness this tolerance of change to generate lantibiotic derivatives with enhanced functionalities. Site directed mutagenesis of lantibiotics was employed for the first time in 1992 (Kuipers et al., 1992, Liu and Hansen, 1992) and has since produced a plethora

of information concerning residue function, composition and enzyme activity, which has been invaluable to advancements in lantibiotic engineering and, indeed, lantibiotic research in general. The first example of the bioengineering of a lantibiotic to enhance activity relates to the nisin-like lantibiotic, subtilin (Liu and Hansen, 1992). Subtilin is a type 1 lantibiotic produced by *Bacillus subtilis* and the bioengineering thereof was facilitated by the replacement of the *spaS* gene on the chromosome by an engineered version using double cross-over homologous recombination. Using this approach, a mutant, in which the fourth residue, glutamate, was replaced with isoleucine, displayed enhanced activity with respect to preventing the spore outgrowth of *Bacillus cereus* T spores.

Similar strategies have been employed on a number of occasions since this time which, although being a relatively time-consuming process, can result in a strain that can be regarded as being non-GM (genetically modified) once used in a contained manner (Sybesma et al., 2006; see http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:125:0075:0097:EN:PDF) *In trans* complementation and heterologous production approaches have also been frequently employed and can be more rapid but result in the strains losing their non-GM status. While these strategies have been employed to generate a considerable number of lantibiotic derivatives, many of which have been of considerable fundamental value (Cortes et al., 2009, Cotter et al., 2005a, Field et al., 2010), here we focus solely on those novel engineered peptides that exhibit enhanced functionalities. In this regard, a number of studies have highlighted the merits of manipulating a three amino acid stretch (Asn20-

Met21-Lys22) located at the centre of the nisin propeptide (NisA), which functions as a hinge around which the receptor binding N-terminus and pore-forming C-terminus rotate. Site-specific mutagenesis has shown that this hinge region plays a vital role in affording nisin the conformational plasticity required for antimicrobial activity (Yuan et al., 2004). Changes to this region conferred properties including improved stability at higher temperatures, and neutral or alkaline pH, in addition to greater solubility. Furthermore, two peptides with enhanced potency (N20K nisinZ and M21K nisinZ) against Gram negative targets *Shigella*, *Pseudomonas* and *Salmonella*, were identified. Unfortunately, however, these mutants displayed reduced activity compared to the wild type peptide against non-pathogenic Gram positive targets such as *Micrococcus flavus* and *Streptococcus thermophilus*, (Yuan et al., 2004) (**Figure 3**). Although the activity of these peptides is below that required for commercial and clinical use or is against non-pathogenic targets, these findings were nonetheless of great significance.

The importance of these hinge residues has also been highlighted using a non-targeted approach, i.e., from screening of a large bank of producers (8,000) of randomly altered nisin peptides (altered to ensure a frequency of 1-3 mutations within the gene; Field et al., 2008). Although a similar approach had been taken previously (Spee et al., 1993), the bank of strains created on that previous occasion was relatively small. Following extensive randomization (through use of a DNA polymerase that incorrectly incorporated nucleotides during PCR), Field and co-workers specifically looked for producers exhibiting enhanced activity against pathogens, and ultimately coupled this approach with

site-directed and site-specific saturation mutagenesis to uncover a number of peptides with improved activity against Gram positive pathogens of clinical or food relevance. More specifically, initial screening revealed that a strain producing a nisin variant in which a K22T change had occurred in the hinge region displayed increased activity against the mastitic pathogen *Streptococcus agalactiae*. This prompted further site-directed and site-saturation mutagenesis of the three hinge residues. Site-saturation mutagenesis is an approach whereby a bank of derivatives (or producers thereof) is created in which the amino acid located at a particular location in the antimicrobial is changed to each of the other 19 natural amino acids, typically through the use of degenerate PCR primers. The combined use of site-directed and site-saturation mutagenesis led to the identification of a number of additional 'enhanced' peptides including Nisin N20P, Nisin M21V and Nisin K22S (Field et al., 2008) (**Figure 3)**.

Similarly, Rink *et al*. showed that ring A mutants KFI and KSI were more potent than nisin A against *Lactobacillus johnsonii* and *Leuconostoc mesenteroides*, and *L. lactis* and *L. johnsonii*, respectively (Rink et al., 2007b). Interestingly, Kuipers and co-workers demonstrated how the substitution of threonine in a double mutation simultaneously generated two mutants, G18Thr and a dehydrated form, G18Dhb. The M17Q/G18Thr mutant displayed increased activity against *Micrococcus flavus*, while M17Q/G18Dhb showed similar activities to nisin Z. Additionally, it was shown that a T2S mutant had increased activity against *M. flavus* and *Streptococcus thermophilus* (Kuipers et al., 1996, Kuipers et al., 1992).

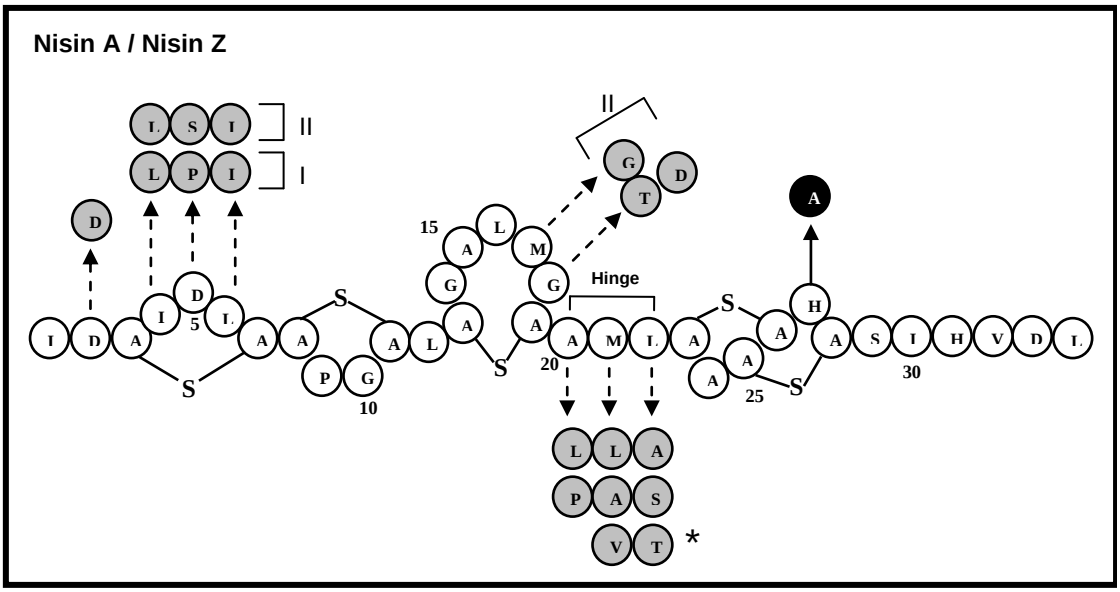

Figure 3

Mutations of Nisin A/Nisin Z resulting in derivatives with enhanced antimicrobial activity. Nisin A and Nisin Z differ by one amino acid, where Nisin Z contains an asparagine residue instead of a histidine at position 27. Positions 20-23 are the “Hinge Region”.

I = Triple mutation, ITL replaced with KFI. (Rink et al., 2007b)

II = Triple mutation, ITL replaced with KSI.(Rink et al., 2007b)

III = Double mutation, MG replaced with QT. Thr was also dehydrated to yield Dhb (Kuipers et al., 1996)

* Numerous other derivatives showed enhanced activity from mutations in this region, but were not investigated further (Field et al., 2008)

Site-saturation mutagenesis has also been successfully employed on a number of other occasions. Such an approach was applied to the bioengineering of the *Staphylococcus warneri* ISK-1-produced type 2 lantibiotic, nukacin ISK-1 (Islam et al., 2009). During this process of mutagenesis, two variants, D13E and V22I, with twice the potency of the wild-type were identified via colony overlay assays, albeit against non-pathogenic strains (such as *Lactobacillus sakei*, *Bacillus coagulans*, *Pediococcus pentococcus* and *Enterococcus faecalis*). Important information regarding the importance of positive charges and ring structures was also obtained during this process. A similar strategy has been utilized in the case of another type 2 lantibiotic, mersacidin (Appleyard et al., 2009). Although a system to facilitate the bioengineering of mersacidin had been previously identified (Szekat et al., 2003), this was improved upon to facilitate the generation of large numbers of variants (Appleyard et al., 2009). The *trans* complementation system in question utilizes an inactive *mrsA* (structural peptide-ending gene) mutant of the producing strain, which is complemented through the introduction of a shuttle plasmid carrying the *mrsA* gene, or a derivative thereof. A simplified transformation procedure to deliver the plasmid to the host by electroporation of demethylated DNA was developed to facilitate the process. It was noted that of the 228 mersacidin mutants in the saturation mutagenesis library, >80 mutants produced mature mersacidin at acceptable levels. 6 variants, G8H, G9S, G10A, G10N, G10V and G10Y, showed increased activity against both MRSA and vancomycin resistant enterococci (VRE), while another 9, P6H, G7A, G7N, G8N, G8Q, G9A, G9H, L14V and S16A, displayed increased activity against VRE only.

While the 'novel' lantibiotics described above differ quite subtly from the existing antimicrobials, the lantibiotic biosynthetic machinery can also be harnessed *in vivo* or *in vitro* to facilitate the creation of peptides which differ more significantly. In one case, Levengood *et al*. employed an approach whereby a biosynthetic enzyme was used to modify synthetic substrate analogues via a strategy termed *in vitro* mutasynthesis (IVM) (Levengood et al., 2009). More specifically, they showed that LctM, the modification enzyme for the type 2 lantibiotic lacticin 481, continued to modify residues within the lacticin 481 propeptide even when residues (Trp19 and Phe23) were substituted with nonproteinogenic amino acids (naphthyalanine and homophenylalanine, respectively). This resulted in the creation of two analogues with increased biological activity against *L. lactis* HP and *Bacillus subtilis* ATCC 6633, thereby demonstrating the value of this approach. Indeed, it has been established that many lantibiotic biosynthetic proteins can be harnessed (Kuipers et al., 2004, Li et al., 2006, van Saparoea et al., 2008, Rink et al., 2007a, Rink et al., 2005, Kluskens et al., 2005, Chatterjee et al., 2006, Kuipers et al., 2008), which will undoubtedly facilitate the synthesis of even greater numbers of novel antimicrobials in the future. Indeed, the application of modification proteins and *in-silico* screening have been nicely combined by Majchrzykiewicz and co-workers who successfully utilized the nisin expression/modification system to produce, modify, and secrete entirely unrelated putative lantibiotics identified using BAGEL (Majchrzykiewicz et al., 2010). The two putative structural peptides of the potentially novel type 2 lantibiotic pneumococcin, A1 and A2, from *Streptocccus pneumoniae* R6, were

chosen as the substrates for the nisin enzymes. Their propeptide regions (i.e. mature peptide region) were fused with nisin leader sequences and introduced into a *Lactococcus lactis* host that overproduces NisBTC. The peptides produced were shown to be modified and to exhibit biological activity against *Micrococcus flavus*. It is thus apparent that the nisin modification and transport machinery can be employed to harness a putative lantibiotic-encoding gene cluster corresponding to a different lantibiotic type and from a different genus and thus could potentially be employed to access the many other putatively lantibiotic-encoding gene clusters referred to above.

1.8 Non-lantibiotic, ribosomally synthesized, modified peptides

As noted above, this review has focused specifically on lantibiotic-related research with a view to using the developments in this area to highlight the variety of different ways in which modified ribosomally synthesised antimicrobials in general can be identified. However, to highlight the relevance of these approaches to other gene-encoded peptides, a selection of recent examples of note is presented here.

1.8.1 Sactibiotics

Clostridium difficile is the causative agent of nosocomial diarrhea, and *C. difficile*-associated disease is increasing in both prevalence and severity. The main predisposing factor for this disease is antibiotic therapy, which often eradicates beneficial flora in the gut, allowing *C. difficile* to flourish. To this effect, a bio-screen was devised with the aim of isolating a narrow-spectrum

bacteriocin effective against *C. difficile* that would not impact on beneficial microbes in the intestine (Rea et al., 2010). It was hypothesised that spore-forming, anaerobic bacteria would be a probable source of bacteriocins active against a related bacterium such as *C. difficile*. To select for such strains, human faecal samples from healthy and diseased adults were treated with ethanol for 30 minutes to kill all vegetative cells. These were then plated on Wilkens Chagrin anaerobic agar (WCAA) and allowed to grow for 5 days at 37^{o}C in an anaerobic chamber. Resulting colonies were overlaid with *C. difficile*-inoculated reinforced *Clostridium* agar and grown for another 18 hours, to produce a lawn of *C. difficile* growth. The plates were then inspected for zones of clearing, where the initial colony inhibited the growth of *C. difficile*. 30,000 colonies were screened, and only one colony showed potent antimicrobial activity against the overlaid *C. difficle* strain. Interestingly, other faecal bacteria growing in the bottom layer were not inhibited by this antimicrobial suggesting it could be a narrow spectrum antimicrobial. The producing colony in question was removed from the agar using a sterile scalpel and sub-cultured onto fresh WCAA. Proteinase tests were performed to confirm that the inhibitory substance was proteinaceous in nature and it was ultimately established to be a two peptide bacteriocin, designated thuricin CD, that underwent post-translational modification resulting in the formation of **s**ulphur to **α** **c**arbon linkages (from which the name sactibiotic is derived) (Rea et al., 2010). The thuricin CD gene cluster was identified through reverse genetics and inverse PCR and was found to contain two radicial SAM (S-adenosyl methyltransferase) encoding genes, *trnC* and *trnD* (Rea et al., 2010). An *in-silico* screen for novel sactibiotics, using

the radical SAM sequences as drivers, as uncovered a considerable number of additional gene clusters of note (Murphy et al., 2011).

1.8.2 Labyrinthopeptins

The labyrinthopeptins are a novel family of lantibiotic-like antimicrobials which contain an unprecedented carbacyclic, post-translationally modified amino acid named labionin (Meindl et al., 2010). These were identified when the culture extracts of a newly identified novel actinomycete *Actinomadura namiensis* DSM 6313 (from Namibian desert soil) were shown to have moderate activity against the herpes simplex virus. The peptide was isolated using chromatographic methods and shown to have potential applications in the treatment of neuropathic pain.

1.8.3 TOMMs

The Thiazole/Oxazole-Modified Microcins (TOMMs) are a group of post-translationally modified antimicrobial peptides, that includes assorted bacterial products such as microcins, thiopeptides, cyanobactins, putative *Bacillus*-associated thiazole–containing heterocyclic bacteriocins, a nitrile hydrolase and Nifl 1 related precursor family, and which are grouped on the basis of containing thiazole and oxazole structures (Molloy et al., 2011). The culture-, *in-silico*- and bioengineering-based approaches described in this review can also be employed to identify novel such peptides. By way of example, we will focus on the thiopeptides.

Thiopeptides are another distinct group of ribsosomally, post-translationally modified antimicrobials (Bagley et al., 2005). Thiopeptides are complex, highly modified sulfur-containing peptides which inhibit the initial steps of protein synthesis in Gram positive bacteria, including MRSA. They contain a macrocyclic framework consisting of modified heterocyclic residues, including indoles, oxazoles, thiazoles and dehydroamino acids. The development of screening programs has greatly expanded the number of known thiopeptide antibiotics in recent years. Although micrococcin P_1 was the first thiopeptide antibiotic to be discovered (Su, 1948), thiostrepton has been the most extensively studied. Thiostrepton exhibits activity analogous to that of penicillin, but has not yet been developed for clinical use since bacterial resistance develops before a therapeutic dose can be reached, due to its low solubility (a problem common to many thiopeptide antibiotics).

Targeted screening programs have isolated a number of thiopeptides from a variety of actinomycete sources. The chemical structure of several thiopeptides has been elucidated using X-ray crystallography and NMR techniques, and several, such as promothiocin A, amythiamicin D and thiostreptin have been synthesised chemically. These results offer a glimpse at a promising future in which chemical thiopeptide structures can be computationally and biologically optimised for antimicrobial activity. A high-throughput screening strategy has been employed, using 96- and 384-well microtiter plates to screen a library of thiopeptide precursor compounds for their abilities to inhibit translation or reverse the inhibition of known thiopeptide antibiotics, to identify four distinct classes of precursor peptides

(Starosta et al., 2009), an *in-silico* screen was used to identify thiocillin, a thiopeptide that undergoes 13 post-translational modifications (Brown et al., 2009) and TP-1161, a thiopeptide antibiotic from a marine *Nocardiopsis* species, was identified by PCR screening (Engelhardt et al., 2010).

1.9 Conclusion

There are numerous strategies available when targeting the identification/creation of novel post-translationally modified antimicrobials. Great advances have been made and today culture-based methods have evolved to encompass high-throughput screening, molecular tools to amplify and engineer DNA and *in-silico* databases and search tools enable bioinformatic mining for novel peptides. The continued evolution of these technologies will ensure that the rate of identification of novel modified antimicrobials will continue to increase at a considerable rate.

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CHAPTER II

Traditional fermented beverages with health-promoting potential: current and future perspectives

2.1 ABSTRACT

Fermentation is an ancient form of food preservation and is also employed as a means of improving the nutritional content of foods. Many such foods, including fermented beverages, have become known for their health-promoting attributes including, in some cases, the treatment/prevention of gastrointestinal ailments. Today, most of the commercial fermented beverages that are regarded as health-promoting are dairy-based. As a consequence of the perception that these beverages contain health-promoting microbes, or because they can be used as a vehicle for specific probiotic strains, these bio-active beverages are increasing in popularity. Examples include fermented dairy-based beverages such as kefir, koumiss, and a variety of African fermented milks, cereal-based beverages such as boza, togwa, kvass and amazake, and other forms of fermented beverages such as kombucha, water kefir and hardaliye. In addition to harnessing traditional beverages, there have recently been innovative efforts to develop non-dairy probiotic fermented beverages from a variety of substrates, including soy milk, whey, cereals and vegetable and fruit juices. Efforts have also been made to enhance these and other beverages through the addition of flavours, prebiotics and nutraceuticals. On the basis of recent developments, it is anticipated that fermented beverages will continue to be a growing sector within the functional food market.

2.2 Introduction

Societies throughout the world independently discovered the value of fermenting food as a cheap means of preservation, improving nutritional quality and enhancing sensory characteristics. The fermentation of milk, cereals and other substrates to produce beverages with health-promoting properties is indigenous to many regions, including Asia, Africa, Europe, the Middle East and South America. Evidence from pottery vessels show that fermented rice, honey and fruit beverages date as far back as 7000 B.C. in China (McGovern et al., 2004), cacao beverages have been dated to before 1000 B.C. from the Honduras region (Henderson et al., 2007), while it is known that kombucha was manufactured by approximately 220 B.C. (Dufresne and Farnworth, 2000).

The global functional beverage market is a growing sector of the food industry as modern health-conscious consumers show an increasing desire for foods that can improve well-being and reduce the risk of disease (Landstrom et al., 2007). Fermented milks, especially yoghurt-style products, are the most popular functional beverages with kefir in western Europe and North America and ymer in Denmark being good examples. Notably, the global functional food and drink market increased 1.5 fold between 2003 and 2010, and is expected to grow a further 22.8% between 2010 and 2014 to be worth $29.8 billion (Leatherhead, 2011). It is also intriguing to note that a number of food companies that have been under pressure, due to the poor public perception regarding the 'healthiness' of the foods they produce, are now focusing on developing functional products (Hugenholtz, 2013). Here we review the

literature regarding traditional fermented beverages with reputed health benefits, and new developments in this field.

2.3 Natural fermented beverages: sources and microbial composition

2.3.1 *Fermented milk beverages*

Many communities across the world produce fermented milks (most notably in Africa, Asia, Europe and the Middle East) with many of these products being of a yoghurt-style consistency. Fermented milk products can be made with milk (or skimmed milk) from various sources, including cow, camel, goat, sheep, yak, and even coconut, milk, and can be either pasteurised or unpasteurised. They can be produced through the use of defined starter cultures, back-slopping or allowed to ferment naturally. Although fermented milk beverages are predominantly composed of lactic acid bacteria (LAB) (Tillisch et al.), the exact microbial content may vary depending on the source of milk, treatment of the milk (e.g. pasteurisation), use of starters, the nature of the local environmental microbes present, temperatures, hygiene, the type and treatment of containers used, and the length of fermentation (Feresu and Muzondo, 1989, Mutukumira, 1995). Many artisanal fermented milk beverages are produced as a result of back-slopping (Mutukumira, 1995), whereby a small portion of already-fermented milk is used to begin a new fermentation. In this way, cultures from the LAB naturally present in the raw milk are passed from household to household and between generations. While the consumption of spontaneously fermented milk is common to many different regions, the exact microbial differences between these products has not been ascertained. **Table 1** lists a

number of the most popular and best-studied fermented beverages from around the world, along with information with respect to their corresponding microbial compositions. From this, the domination of milk-based beverages fermented by LAB, mainly *Leuconostoc*, lactobacilli and lactococci, is clear. Fermentation in colder climates promotes the growth of mesophilic bacteria such as *Lactococcus* and *Leuconostoc*, whereas beverages produced at higher temperatures usually have greater counts of thermophillic bacteria such as *Lactobacillus* and *Streptococcus* (Tamime and Robinson, 1988). The contributions of slime-producing species (e.g. *Leuconostoc* spp.) or acetic acid producing species, generally present at low abundance relative to *Lactobacillus* or *Lactococcus* species, vary depending on abundance. There may also be significant numbers of coliforms present, depending on the level of hygiene employed during preparation. The quantity and types of yeasts involved can vary greatly, but *Candida* and *Saccharomyces* spp. are most commonly detected.

Of the many fermented milk beverages, kefir has been a notable success, gaining worldwide popularity. This drink dates back over 2000 years to the shepherds of the Caucasian region who noticed that the spontaneous fermentation of milk carried in leather pouches preserved the milk over long periods of travel. Over time, kefir became known for its positive impact on the gastrointestinal tract of the consumer (Metchnikoff and Metchnikoff, 2004).

Table 1 A compilation of various milk, cereal and other fermented beverages popular around the world, with their corresponding microbial populations and substrates.

Product	Substrates	Region	Microflora	References
Amasi	Milk (Cow, Various)	Africa (Zimbabwe)	Lactococus (*L.lactis*), *Lactobacillus*, *Leuconostoc*, *Enterococcus*. Uncharacterised fungal component	(Osvik et al., 2013)
Aryan	Milk (Cow, Various)	Turkey	*Lactobacillus bulgaricus*, *Streptococcus thermphilus*	(Altay et al., 2013)
Garris	Milk (Camel)	Africa (Sudan)	LAB (*Lactobacillus* (*L. parachesei*, *L. fermentum* and *L. plantarum*), *Lactococcus*, *Enterococcus*, *Leuconostoc*). Uncharacterised fungal component	(Sulieman et al., 2006)
Kefir	Milk (Cow, Various)	Eastern Europe (Caucasian region)	*Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Acetobacter* ; *Naumovozyma*, *Kluyveromyces*, *Kazachstania*	(Marsh et al., 2013c)
Kivuguto	Milk (Cow)	Africa (Rwanda)	LAB (*Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides* and *Lactococcus lactis*). Uncharacterised fungal component	(Karenzi et al., 2013)
Koumiss/Airag	Milk (Cow, Camel, Goat)	Asia/Russia	LAB (predominantly *Lactobacillus*) and yeast (*Kluyveromyces*, *Saccharomyces* and *Kazachstania*)	(Mu et al., 2012, Hao et al., 2010)
Kumis	Milk (Cow)	South America (Columbia)	LAB (*Lactobacillus cremoris*, *Lactococcus lactis*), *Enterococcus* (*E. faecalis*, *E. faecium*) Yeast (*Galactomyces geotrichum*, *Pichia kudriavzevii*, *Clavispora lusitaniae*, *Candida tropicalis*)	(Chaves-Lopez et al., 2011, Chaves-Lopez et al., 2012, Gaviria et al., 2010)
Nyarmie	Milk (Camel)	Africa (Ghana)	LAB (*Leuconostic mesenteroides*, *Lactoabcillus bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus lactis*, *Lactococcus lactis*);	(Obodai and Dodd, 2006)

			Saccharomyces cerevisiae	
Rob	Milk (Unspecified)	Africa (Sudan)	LAB (*Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Lactococcus lactis*, *Streptococcus salivarius*); Yeast (*Saccharomyces cerevisiae*, *Candida kefyr*)	(Abdelgadir et al., 2001)
Suusac	Milk (Unspecified)	Africa (Kenya)	LAB (*Leucontostoc mesenteroides*, *L. plantarum*, *L. cruvatus, L. salivarius, L. raffinolactis*); Yeasts (*Candida krusei*, *Geotrichum penicillatum*, *Rhodotorula mucilaginosa*)	(Lore et al., 2005)
Amazake	Rice	Japan	Fungi (*Aspergillus* spp)	(Saigusa and Ohba, 2007, Yamamoto et al., 2011)
Boza	Various (Barley, Oats, Rye, Millet, Maize, Wheat or Rice)	Balkans (Turkey, Bulgaria)	*Leuconostoc* (*L. paramesenteroides, L. sanfrancisco, L. mesenteroides*), *Lactobacullus* (*L. plantarum, L. acidophilus, L. fermentum*); *Saccharomyces* (*S. Uvarum, S. Cerevisiae*), *Pichia fermentans, Candida spp.*	(Botes et al., 2007, Gotcheva et al., 2000, Hancioglu and Karapinar, 1997, Caputo et al., 2012)
Bushera	Sorghum, Millet flour,	Africa (Uganda)	*Lactobacillus*, *Streptococcus*, *Enterococcus*. Uncharacterised fungal component	(Muyanja et al., 2003)
Koko Sour Water	Cereal (Pearl Millet)	Africa (Ghana)	LAB (*Weisella confuse*, *Lactobacillus fermentum*, *Lactobacillus salivarius*, *Pediococcus* spp.) Uncharacterised fungal component	(Lei and Jakobsen, 2004)
Kvass	Rye bread, rye and barley malt/flour,	Russia	LAB (*Lactobacillus casei*, *Leuconostic mesenteroides*); *Saccharomyces cerevisiae*	(Dlusskaya et al., 2008)
Mahewu	Maize, Sorghum/Millet	Africa (Zimbabwe)	Unknown	(Mugochi et al., 2001, Gadaga et al., 1999)
Pozol	Maize	Mexico (Southeast)	LAB (*Lactococcus lactis*, *Streptococcus suis*, *Lactobacillus* (*L. plantarum*, *L. casei*, *L. alimentarium*, *L. delbruekii*), non-LAB (*Bifidobacterium*, *Enterococcus*). Uncharacterised fungal component	(Ampe et al., 1999, ben Omar and Ampe, 2000, Escalante et al., 2001)
Togwa	Maize flour, Finger Millet	Africa (Tanzania)	*Lactobacillus*, *Saccharomyces cerevisea*,	(Mugula et al.,

	Malt,		*Candida* spp.	2003, Kitabatake et al., 2003a)
Hardaliye	Grapes/Mustard Seeds/Cherry Leaf	Turkey	*Lactobacillus*. Uncharacterised fungal component	(Altay et al., 2013)
Kombucha	Tea	China, Worldwide	*Gluconacetobacter*, *Lactobacillus*; *Zygosaccharomyces*	(Marsh et al., 2013a)
Water Kefir	Water/Sucrose	Mexico, Worldwide	*Lactobacillus*, *Zymomonas*; *Dekkera*, *Hanseniaspora*, *Saccharomyces*, *Zygosaccharomyces*	(Marsh et al., 2013b)

The microorganisms responsible for the fermentation are actually a symbiotic combination of bacteria and yeast, contained within a polysaccharide matrix, known as kefir 'grains' (Farnworth, 2006). So complex is the symbiotic relationship, that *in vitro* synthesis of kefir grains has yet to be replicated. Therefore, commercial kefir is produced by defined starters, and probiotic strains are also added to some products.

Koumiss, sometimes known as airag (a name usually indicating it is produced from mare's milk), is a popular beverage of nomadic cattle breeders in Asia and some regions of Russia. Like kefir, it is fermented by a symbiotic combination of microbes to produce a sour, sparkling beverage. However, unlike kefir, there is no solid inoculation matrix, and this milk is fermented by back-slopping or by allowing the milk to ferment naturally. The microorganisms responsible for both products are alike, but for the fact that koumiss has been reported to contain fewer lactococci. Both kefir and koumiss have become popular worldwide due to their reputed ability to improve gastrointestinal health (Jagielski, 1877, Saijirahu, 2008, Metchnikoff and Metchnikoff, 2004). Kumis is a similarly prepared beverage that is found in Colombia, which has been shown to contain significant proportions of enterococci, an observation which has been attributed to poor hygiene (Chaves-Lopez et al., 2011).

In Africa, fermented milk beverages are quite popular, and the art of making fermented products is passed down through generations (Caplice and Fitzgerald, 1999). Examples of such beverages include amasi from Zimbabwe, kivuguto from Rwanda, suusac from Kenya, nyarmie from Ghana and rob and garris from Sudan (Obodai and Dodd, 2006, Lore et al., 2005, Osvik et al., 2013,

Karenzi et al., 2013, Abdelgadir et al., 2001, Sulieman et al., 2006). Considering that most of these are derived from the spontaneous fermentation of milk by its innate flora, it is likely that the fermented milks, although known by different names are actually quite similar (in the same way airag and koumiss may be considered similar), and have, in combination, been referred to as naturally fermented milk (NFM) (Narvhusa and Gadaga, 2003). Exceptions exist in that amasi, the Zimbabwean fermented milk has a distinct sensory profile (Narvhusa and Gadaga, 2003). Nonetheless, accurate categorization remains difficult in the absence of more detailed microbiological and biochemical analyses.

The aforementioned amasi is fermented in a smoked, calabash container for 3-5 days. Whey is removed until the beverage reaches the desired viscous consistency (Osvik et al., 2013). For nyarmie, the milk is pasteurised and fermented in an aluminium container for 24-48 hours (Obodai and Dodd, 2006). Suusac is prepared by the natural fermentation of camel milk in smoke-treated gourds for 24-48 hours, producing a beverage with low viscosity and distinct smoky taste. The dominant microorganisms in this beverage were found to be Leuconostoc mesenteroides and Candida krusei, with Lactobacillus plantarum also present (Lore et al., 2005). Garris is another fermented camels milk, that is produced by a continuous batch fermentation in large leather pouches and agitated by the movement of the camel (Sulieman et al., 2006). Kivugoto is a naturally fermented beverage produced from unpasteurised cows milk, which takes 2-3 days to produce (Karenzi et al., 2013). Fermentation of rob is started through the transfer of cultures from the previous day's fermentation, and is made in either a goatskin container or gourd made from hollowed dried fruit

(Abdelgadir et al., 2001). Depending on the level of hygiene used in production, contamination with non-milk microbes is frequent. This has been particularly evident in African beverages (Gran et al., 2003).

Finally, in many countries, yoghurts are diluted with water to form drinkable fermented milk. In the Middle East, doogh is very popular and seasoned with mint (Shab-Bidar et al., 2011), while ayran is popular in Turkey (Caglayan et al., 1989). Other examples include chaas and lassi, Indian yoghurt-beverages that are mixed with spices and, on occasion, fruit. The microbial comparison of these beverages is generally quite similar to that of yoghurts.

2.3.2 *Cereal and other fermented beverages*

Another important class of fermented beverages are those made from cereals, which are popular in tropical regions and on the continent of Africa in particular. As with many milk-based products, the natural microbial component is used to ferment grains including maize, millet, barley, oats, rye, wheat, rice or sorghum. The grains are often heated, mashed and sometimes filtered. Back-slopping is again quite common, but the microbial populations responsible for the fermentation of these beverages are not as well characterised.

Boza, consumed in Bulgaria and Turkey, is generated through the fermentation of a variety of cereals including barley, oats, rye, millet, maize, wheat or rice, with the specific composition affecting the viscosity, fermentability and content of the final beverage (Akpinar-Bayizit et al., 2010). The cereal is boiled and filtered, a carbohydrate source is added, and the mixture can be left to ferment independently or with the use of back-slop. Boza

has yet to be commercialised and studies have revealed that the microbial population varies (Botes et al., 2007, Gotcheva et al., 2000, Hancioglu and Karapinar, 1997). The function of the yeast present, which are only sometimes detected, remains unknown. Of several combinations, fermentation by *S. cerevisiae*, *Leuconostoc mesenteroides* and *Lactobacillus confusus* was shown to produce the most palatable beverage (Zorba et al., 2003).

Togwa, a sweet and sour, non-alcoholic beverage, is one of the better studied African cereal beverages. Produced from the flour of maize, sorghum and finger millet and sometimes, cassava root, the chosen substrates are boiled, cooled and fermented for approx. 12 hours to form a porridge, which is then diluted to drink (Kitabatake et al., 2003b). Mahewu is similar in that maize or sorghum meal is fermented with millet or sorghum malt, and is available commercially (Mugochi et al., 2001). Bushera is generally prepared from germinated or non-germinated sorghum grains, and fermented for 1-6 days (Muyanja et al., 2003). These beverages are often used to wean children, and as a high-energy diet supplement. Koko sour water is the fermented liquid water created in the production of the fermented porridge, koko. This contains a high portion of LAB and is used by locals to treat stomach aches and as a refreshing beverage (Lei and Jakobsen, 2004).

Kvass is a fermented rye bread beverage common in Russia, which has seen much commercial success (Dlusskaya et al., 2008). The beverage can have a sparkling, sweet or sour, rye bread flavour. Its alcohol content, though usually low, can vary, and has been suggested as a contributor to alcoholism (Jargin, 2009). Amazake is a sweet fermented rice beverage that is the non-alcoholic

precursor to sake, produced in Japan. Steamed rice is mixed with rice-koji (Aspergillus-mycelia and rice) and water, and is heated to 55-60°C for 15-18 hours. Enzymes break down the rice and form glucose content of approximately 20%. Amazake is highly nutritious and is consumed for its purported health benefits (Yamamoto et al., 2011, Saigusa and Ohba, 2007). Pozol, which is common to south-eastern Mexico, has a quite different method of production, in that maize grains are heat-treated in an acid solution, ground and shaped into dough balls. These are then wrapped in banana leaves and fermented for 2-7 days, after which they are resuspended in water and consumed as beverages. Pozol is composed of a variety of microorganisms including LAB, non-LAB, yeasts and other fungi (Ampe et al., 1999, ben Omar and Ampe, 2000, Escalante et al., 2001).

In addition to milk and cereal-based fermentations, there are also other forms of fermented beverages. One example is kombucha, which is a fermented sweetened tea that was originally popular in China but is now enjoyed worldwide. It is fermented by a symbiotic mixture of bacteria (typically acetic acid bacteria, with small quantities of LAB) and yeast, which are embedded within a cellulosic matrix that floats above the fermentate, similar to the mother cultures of vinegar. Due to the high acid content (as low as pH2), the functionality of kombucha is predominantly due to its physiochemical properties (Greenwalt et al., 2000). As a result of the tea content, it also contains a number of phenols and vitamins (Dufresne and Farnworth, 2000). Water kefir is similar in concept to milk kefir in that it is fermented by a symbiosis of bacteria and yeast contained within grains. However, these grains

are composed of dextran, are translucent and crystal-like in appearance, and are thought to have originated in Mexico where they formed as hard granules fermented from sap on the pads of the *Opuntia* cactus. They ferment sweetened water, to which figs and lemon are traditionally added for additional flavour and nutrients. The composition of water kefir can vary, but is known to contain LAB, including *Lactobacillus*, and *Bifidobacterium* (Gulitz et al., 2013, Marsh et al., 2013b). Hardaliye is a non-alcoholic, Turkish, fermented beverage made from red grapes, black mustard seeds, cherry leaf and benzoic acid. Ingredients are pressed and fermented for 5-10 days at room temperature. Again, the microbial population has been reported to be predominantly composed of *Lactobacillus* and unknown fungal components (Altay et al., 2013), and this beverage is thought to have antioxidant properties (Amoutzopoulos et al., 2013).

2.4 Beyond physiochemical advantages: from microbial content to functionality

2.4.1 *Molecular-based microbial characterisation*

Despite the data presented above, many of these beverages have not been the focus of detailed microbiological analyses. Some molecular-based microbial characterisation of these beverages have taken place and have mostly relied on low-throughput approaches, employing techniques such as DGGE, which can only assess 1-2% of a population (Muyzer et al., 1993).

Moving forward, the availability of molecular technologies such as culture-independent high-throughput sequencing based microbial analyses,

metabolomics and bioinformatics will prove particularly useful, and will provide a more accurate picture of these populations, surmounting problems associated with relying on phenotypic-based approaches (Giraffa and Neviani, 2001). In-depth molecular studies have the potential to be particularly useful when carrying out analyses across different beverages with a view to attributing specific desirable or non-desirable sensory characteristics with specific microorganisms present (Marsh et al., 2013c, Marsh et al., 2013b). Such approaches will also ultimately facilitate accurate species identification, leading to novel starter design, and the development of beverages with different and complex flavour profiles. It will also be possible to more effectively monitor the change of proportions of different species throughout fermentation and storage (Cocolin et al., 2013).

2.4.2 *Health-promoting microbes*

It is widely believed that the primary reason for the functionality of these beverages is due to the presence of specific live microorganisms. To the consumer, health claims are more important than nutritional claims (Verbeke et al., 2009), so there has and will be a desire to augment the health-promoting potential of these beverages through the addition of certified probiotics. The WHO/FAO defines probiotics as “live microorganisms, which when administered in adequate amounts confer a health benefit on the host” (Araya et al., 2002), and the probiotic sector is the largest component of the functional food market. The physiology of certain strains of lactobacilli and bifidobacteria make them well-suited to both the gastrointestinal and milk environments, and thus lactic

acid bacteria and bifidobacteria are the most studied and utilised probiotic organisms. It is generally considered that a minimum of 109 cells per daily dose are required for probiotics to be effective (Forssten et al., 2011). Within the EU, the term “probiotic” is now considered a health claim, and most applications submitted to the European Food Safety Authority (EFSA) have been rejected (Guarner et al., 2011, Schmidt, 2013). In Europe, boosting numbers of *Lactobacillus* and *Bifidobacterium* in the gut is not deemed to be of sufficient merit to be considered a health benefit; the link must be made to a physiological (e.g. strengthening the immune system or resistance to infections) benefit to the host. Proving such health claims is expensive, and in the midst of unclear definitions and guidelines, industries are currently more likely to develop and market probiotic products in other parts of the world (Katan, 2012). In situations where probiotic strains are added during a fermentation, they must not interact antagonistically with starter strains. This becomes less of an issue if strains are added after fermentation is complete, due to the low metabolic rates at refrigerated temperatures (Heller, 2001). Additionally, microencapsulation technology may aid in the delivery of probiotic strains by protecting them in non-native environments. Microencapsulation of *Bifidobacterium* increased viable numbers when added to mahewu, without significantly impacting on flavour, suggesting it could be an effective probiotic delivery system (McMaster et al., 2005).

Many traditional beverages are themselves capable of conferring health benefits, given their historical and frequent use to treat gastrointestinal ailments (Jagielski, 1877, Saijirahu, 2008, Metchnikoff and Metchnikoff, 2004).

However, in most cases, there is insufficient scientific evidence to substantiate the associated health claims. Nonetheless, natural fermented milks have been shown to have antihypertensive effects, enhance systemic immunity, help lower cholesterol and to help lower blood pressure (Gonzalez-Gonzalez et al., 2011, Dong et al., 2013, Galdeano et al., 2011, Wang et al., 2010). In recent human trials, they have been shown to aid in the treatment of IBS and to help alleviate constipation (Simren et al., 2010, Agrawal et al., 2009, Tabbers et al., 2011, Marteau et al., 2013, Matsumoto et al., 2010). Additionally, they have been shown to have modulatory effects on the brain, and demonstrate anti-cancer potential (Kumar et al., 2012, Tillisch et al., 2013). Furthermore, traditional beverages are often reported to contain health-promoting strains, usually in reference to the presence of lactic acid bacteria such as lactobacilli, but this again requires validation. Notably, there are some fermented beverage isolates that have shown promise due to their in vitro gastric survival and cell adhesion, such as lactobacilli from boza (Todorov et al., 2008), *Lactobacillus rhamnosus* strains from fermented mare's milk (Shi et al., 2012) and bacterial isolates from koumiss and other dairy products (Pan et al., 2011, Zhang et al., 2010, Wu et al., 2009). Additionally, the ability to produce antimicrobial substances, believed to contribute to probiotic activity (Dobson et al., 2012), has been demonstrated by various fermented beverage isolates (Powell et al., 2007, Todorov, 2008, Todorov, 2010, von Mollendorff et al., 2006, Batdorj et al., 2006).

The health-related role of yeast in fermented beverages has yet to be fully elucidated. The volume of studies reporting significant numbers of yeast in traditional fermented beverages indicates their importance in these

fermentations. Yeasts in dairy produce contribute desirable aromatic compounds, proteolytic and lipolytic activities and can aid bacterial growth by producing amino acids, vitamins and other metabolites, and contribute to the final composition of the product by producing ethanol and carbon dioxide (Viljoen, 2001). In particular, studies have demonstrated that yeast can exert a positive effect on the abundance of *Lactobacillus* in fermented environments (Karaolis et al., 2013, Gadaga et al., 2001), and this might be a key function in such symbioses. Yeast might also play a role in preventing the proliferation of undesirable species such as Enterobacteriaceae (Mathara et al., 2004). Success has been made in incorporating them in commercial fermented milk products, but excessive gas production during storage can be an issue (Lourens-Hattingh and Viljoen, 2001, Karaolis et al., 2013). *Saccharomyces boulardii* is currently the only recognised probiotic yeast. While yeast only comprise <0.1% of the gut microbiota, they are 10 times larger than prokaryotes and can thus impede colonisation of pathogenic bacteria (Czerucka et al., 2007). Some species of yeasts are common to both fermented beverages and the gut microbiota, such as *Saccharomyces* and *Candida* species, and could be investigated with a view to their contribution to fermentations and optimising health-promoting potential.

2.4.3 *Rational design of starter cultures*

The selection of appropriate starter strains will be key in efforts to accurately reproduce the desirable characteristics of traditional health-promoting beverages for mass production. To faithfully reproduce these beverages and traits, microbes should be sourced from the traditional fermented beverages,

given that these microbes have adapted over thousands of years to their respective environments, and are more likely to function at the appropriate pH, salt concentration, temperature etc. For instance, amylolytic digestion of starch could be considered desirable for fermented cereal production, and isolates from boza and pozol have shown to be capable of this metabolic trait (Petrova et al., 2010, Diaz-Ruiz et al., 2003). Such populations also have a history of safe human consumption. Rational strain selection to produce the correct balance of flavour, aroma, texture, acidification, bitterness, speed of fermentation, and the optimum quantity of organic acid, vitamins and minerals is essential (Di Cagno et al., 2013), as beverages that are too sour or bitter, or contain too much ethanol, will not meet consumers' approval. Over recent years, genetic tools have become available to engineer and select superior starter strains, but legislation currently hinders their industrial use (Hansen, 2002). The inclusion of strains producing antimicrobials, such as bacteriocins, could serve as natual preservatives and help produce a more natural product (Cleveland et al., 2001), while sequential fermentation with yeast, followed by bacteria, could produce a beverage with the desired physiochemical effects, but without biostabilisation issues created by excessive gas production (Kwak et al., 1996).

As stated above, the natural fermentation of beverages involves many different strains of bacteria, and sometimes, yeast. There is an understandable tendency to keep starter cocktails simple but, as traditional beverages show, there are often multiple strains involved, including different species or even microorganisms. From a health perspective, multistrain or multispecies probiotic beverages display greater beneficial effects than monostrain cultures

(Timmerman et al., 2004). Incidentally, there is a lack of studies assessing the effects of combining several natural strains on the physiochemical and sensory characteristics of milk or other functional beverages. Without such information, it is difficult to accurately reproduce the characteristics of the organic beverage with one produced by a defined combination of starters, to match the flavour and properties of the original beverage. This is crucial when marketing beverages to consumers already familiar with the artisanally produced variant of the product, and if wishing to retain the health-promoting characteristics attributed to the original product.

2.5 Development of novel functional beverages

Expanding technological capabilities, especially ingredient exploration and development, has led to increased functional product innovation. The number of new products with functional claims has been growing by approximately 28% per year (Leatherhead, 2011). Consumers' willingness to pay a premium price for fortified products is also a key driver for innovation. While most current functional beverages are aimed at the high-income consumer, there is an argument to be made that those who would benefit most from fermented beverages are from underdeveloped nations, where such beverages could provide a cost-effective means of delivering much-needed nutrition (Van Wyk et al., 2002). Fermented foods are particularly common in Africa, where palates are accustomed to sour foods. Incorporation of reliable probiotic cultures into beverages that are already familiar to Africans could be a way of preventing or treating diarrhoea, which claims the lives of many African children. Additionally,

providing a safe, fermented cereal beverage could help reduce diarrhoea and malnutrition caused by contaminated traditional beverages used in weaning children (Motarjemi et al., 1993). Trends in the developments of fermented milks have been covered elsewhere (Khurana and Kanawjia, 2007, Shiby and Mishra, 2013), so here we will focus primarily on the further development of non-dairy functional beverages.

2.5.1 *New substrates*

The US, Europe and Japan markets account for over 90% of total functional foods, with the majority being functional dairy products. However, non-dairy probiotic delivery has been attracting more attention in recent years, partly due to the success of bio-functional foods and the desire to expand and provide an alternative probiotic choice to conventional dairy-based beverages. Non-dairy probiotic beverages are particularly attractive due to their lack of dairy allergens, low cholesterol content and vegetarian-friendly status (Prado et al., 2008, Rivera-Espinoza and Gallardo-Navarro, 2010). Furthermore, they can be rich in antioxidants, dietary fibre, minerals and vitamins.

To this end, cereal-based beverages could be marketed in response to consumers' awareness of the benefits of high fibre diets. They contain natural prebiotic traits due to the presence of indigestible fibres (Kedia et al., 2007) and the presence of diacetyl acetic acid and butyric acid make them palatable, and furthermore, could be cheaper to produce. Oats, a major source of beta-glucan, which can reduce LDL-cholesterol, are known to function as a prebiotic by boosting bifidobacteria numbers in the gut (Martensson et al., 2005), and have

been investigated with a view to producing synbiotic beverages (Gupta et al., 2010, Guo et al., 2003). A fermented oat drink with two *Bifidobacterium longum* strains was shown to normalise bowel movements in elderly patients (Pitkala et al., 2007). Malt and barley have also been used as beverage substrates (Rathore et al., 2012), with malt proving in that instance to be the better of the two cereals, and also demonstrating better growth of health-promoting strains (Charalampopoulos et al., 2002). Emmer, an ancient European cereal has also shown potential as a functional cereal beverage (Coda et al., 2011).

Soy-based beverages also have the possibility to become more popular due to the fact they contain low cholesterol and saturated fats, and are lactose-free (Wagar et al., 2009, Champagne et al., 2010). They have also been shown to be capable of fermentation by *Bifidobacterium*, which in combination with *Lactobacillus*, can have a positive impact on the ecosystem of the intestinal tract (Cheng et al., 2005). Some varieties are already available commercially (Haelan951®, Rcircle, Jiva™) and have been shown to have beneficial effects on the host (Klein et al., 2006).

Whey is a by-product of the cheese industry which retains 55% of milk nutrients and contains only 0.36% fat, and has the potential for further use in the human diet. In an effort to add value to this waste product, numerous studies have investigated its fermentation by lactic acid bacteria (*Streptococcus* and *Lactobacillus*) to produce a lactic probiotic beverage (Pescuma et al., 2008, Pescuma et al., 2010), and probiotic bacteria have already demonstrated good survival in whey (Drgalic et al., 2005, Ying et al., 2013). Prebiotics have also been successfully incorporated, including oligofructose and inulin (de Castro et al.,

2009b, Drgalic et al., 2005), and hydrocolloid thickening agents added to improve viscosity and mouthfeel (Gallardo-Escamilla et al., 2007).

2.5.2 *Fermented juices*

Fruit juices have considerable market value and consumer acceptance (Sun-Waterhouse, 2011). Already considered a healthy food product, fruit juices are often fortified with vitamins and minerals, in addition to having a high nutrient and antioxidant content, and represent a new method of nutrient and probiotic delivery (Sheehan et al., 2007). Indications show that consumers have a preference for the "healthiest" product over taste (Luckow and Delahunty, 2004). As an increasing number of studies are demonstrating, sugars naturally present in juices can facilitate the growth of cultures with appealing taste profiles. This is true of tomato, pomegranate, pineapple, orange and cashew-apple juice (Yoon et al., 2004, Pereira et al., 2011, Costa et al., 2013, Escudero-Lopez et al., 2013, Filannino et al., 2013). These microbes can impact on physiochemical aspects, such increasing concentrations of flavanones and carotenoids in orange juice (Escudero-Lopez et al., 2013), and have shown good survival rates during storage of the beverages. While the final content of such beverages are quite acidic and best suited to fermentation by probiotic Lactobacillus species (*L. casei*, *L. acidophilus*, *L. plantarum*, *L. parachesei* and *L. delbrueckii*), the use of microencapsulation technology could aid in the delivery of other viable probiotic microorganisms (Champagne and Fustier, 2007). The enrichment of juices with brewer's yeast autolysate before fermentation positively impacts on the nutritional content of the final beverage (Rakin et al.,

2004, Rakin et al., 2007), raising the feasibility of co-fermentation by the right combination of bacteria and yeast (Priya and Munishamanna, 2013). Similar microorganisms have also been shown to successfully ferment various vegetable juices including cabbage, beet, pumpkin, courgette and carrot juices supplemented with prebiotics (Yoon et al., 2005, Yoon et al., 2006, Kohajdova et al., 2006, Nazzaro et al., 2008). This topic has been recently reviewed (Martins et al., 2013). Moreover, there is also an option of combining fruit and vegetable juices with fermented milks (Shiby and Mishra, 2013, Daneshi et al., 2013).

2.5.3 *Application of traditional cultures*

In spite of the wide range of options available when designing novel health-promoting fermented beverages, there will always be an attraction for healthy foods derived from natural processes. Applying the solid inoculation matrices of traditional fermented beverages to new substrates provides a means of generating new beverages while retaining natural microbial populations. The use of kefir grains to continuously ferment whey to produce high quality alcohol has been investigated, and they have also been employed to produce whey and cocoa pulp beverages containing health-promoting strains (Magalhaes et al., 2010, Koutinas et al., 2007, Londero et al., 2012, Puerari et al., 2012). Similarly, the cellulosic pellicle of kombucha has been successfully used to ferment malt, milk and cheese whey (Belloso-Morales and Hernández-Sánchez, 2003, Ilicic et al., 2012, Malbasa et al., 2009, Belloso-Morales and Hernandez-Sanchez, 2003, Cvetković and Markov, 2002).

2.5.4 *Enhancing beverage impact*

Most of the beverages described in this review are still in the early stages of commercial development, and require further extensive sensory, physical and chemical characterisation to develop a palatable flavour profile and viable product before commercialisation. Experiments have shown that the addition to fermented milks of flavour enhancers such as spearmint and oregano had a positive impact on the growth of *Lactobacillus* and *Bifidobacterium* (Marhamatizadeh et al., 2012, Marhamatizadeh et al., 2011), whereas citrus fibres had a positive effect on *Lactobacillus* only (Sendra et al., 2008). Artificial sweeteners were demonstrated to have positive effects on the flavours of an oat-based beverage (Angelov et al., 2006). Prebiotics, including fructooligosaccharides, inulin and galactooligosaccharides, are added commercially to fermented milks to promote the growth of favourable bacteria (Huebner et al., 2007), while investigations of other prebiotics such as oligofructose and polydextrose have also yielded positive results (Oliveira et al., 2009, De Castro et al., 2009a). Enrichment of fermented milk and soy beverages with plant sterols can contribute to controlling hypercholesterolemia (Weidner et al., 2008, Hansel et al., 2007), while fortification of fermented milks with iron improved the growth of preschool children (Silva et al., 2008). In addition to preventing and treating intestinal-associated diseases, the incorporation of nutraceuticals such as ω-3 fatty acids, isoflavones and phytosterols in fermented milks also have potential applications (Awaisheh et al., 2005).

2.6 Conclusion

Fermentation of products is an ancient form of bio-preservation that is common to all regions of the world. With traditional milk-fermented products currently enjoying great success in many markets, there is an increasing interest in functional beverages from a scientific, consumer and commercial perspective. In addition to the many forms of traditional milk-based beverages, there are also other varieties of traditional beverages that could be utilised as alternative methods for probiotic delivery and simply as alternatives to milk-based functional drinks.

These beverages also represent an excellent source for starter strains in industrial fermentations, and for potential probiotic/health-promoting strains. One obvious hurdle is consumers' willingness to accept an unfamiliar product, but with the right combination of starters and substrates, optimum nutrition and flavour development and with scientifically-supported health benefits, the market exists for such products. Indeed, with the availability and improvements in technology, and consumers' increasing interest in functional foods, the outlook for fermented beverages is as promising as it has ever been.

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CHAPTER III

In-silico analysis highlights the frequency and diversity of type 1 lantibiotic gene clusters in genome sequenced bacteria

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3.1 ABSTRACT

Lantibiotics are lanthionine-containing, post-translationally modified antimicrobial peptides. These peptides have significant, but largely untapped, potential as preservatives and chemotherapeutic agents. Type 1 lantibiotics are those in which lanthionine residues are introduced into the structural peptide (LanA) through the activity of separate lanthionine dehydratase (LanB) and lanthionine synthetase (LanC) enzymes. Here we take advantage of the conserved nature of LanC enzymes to devise an *in-silico* approach to identify potential lantibiotic-encoding gene clusters in genome sequenced bacteria. In total 49 novel type 1 lantibiotic clusters were identified which unexpectedly were associated with species, genera and even phyla of bacteria which have not previously been associated with lantibiotic production. Multiple type 1 lantibiotic gene clusters were identified at a frequency that suggests that these antimicrobials are much more widespread than previously thought. These clusters represent a rich repository which can yield a large number of valuable novel antimicrobials and biosynthetic enzymes.

3.2 INTRODUCTION

Bacteriocins are bacterially produced peptide antibiotics. Two major classes of gram-positive bacteriocins have been recognised, Class I undergo significant post-translationally modifications while the Class II are unmodified (Cotter et al., 2005, Rea, 2010). The majority of the class I bacteriocins are lantibiotics; small peptides containing internal bridges resulting from the formation of (β-methyl)lanthionine residues. The structural gene encodes a ribosomally synthesised precursor prepeptide which is generically named LanA. This prepeptide contains a leader sequence at the N-terminus, which is ultimately cleaved, and a propeptide at the C-terminus. Many or all of the serine and threonine residues within the propeptide are dehydrated to form dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. When these modified residues interact with an intrapeptide cysteine, a thioether bond is formed resulting in the formation of lanthionine (Lan, from Dha) or β-methyl lanthionine (meLan, from Dhb).

The lantibiotics and lantipeptides (lanthionine containing peptides which lack antimicrobial activity) can be divided into four groups according to the nature of the enzymes which catalyse (me)Lan formation (Willey and van der Donk, 2007). In the case of type 1 lantibiotics two enzymes are involved; LanB, the lanthionine dehydratase which catalyses the dehydration of the amino acids, and LanC, the lanthionine synthetase which catalyses thioether formation. Type 2 lantibiotics contain a single LanM enzyme which performs both functions. Type 3 and 4 are lantipeptides which are also catalysed by distinct enzymes such as the RamC-like and LanL enzymes (Kodani et al., 2004 ,

Goto et al., 2010). The type 1 and 2 lantibiotics can also be further subdivided on the basis of the amino acid sequence of the unmodified propeptide. In the case of the type 1 lantibiotics, five such subgroups have been identified, each of which is named after the corresponding prototypical lantibiotic; the nisin-like, epidermin-like, Pep5-like, streptin-like and planosporocin-like lantibiotic subgroups (Rea et al., 2010; Piper et al., 2009). The nisin-like group is named for nisin A, which is the most extensively studied bacteriocin and is currently sold in more than 50 countries as a food preservative (Delves-Broughton, 2005). In addition to LanA, B and C, other proteins involved in the production of nisin A and other type 1 lantibiotics include LanP, a serine protease that cleaves the leader from the propeptide; LanT, an ABC transporter responsible for the transport of the lantibiotic precursor across the cell membrane; LanIEFG encode proteins involved in immunity; and LanK, a histidine kinase and LanR, a response regulator, that together operate as a two-component regulatory system. LanD enzymes, such as that responsible for the oxidative decarboxylation of C-terminal cysteines in epidermin (Kupke et al., 1992), are less common.

Given the broad antimicrobial spectrum of many lantibiotics, the possibility of applying lantibiotics in a medicinal capacity has become the subject of much attention. This is supported by an enhanced understanding of their mechanisms of action (Bierbaum and Sahl, 2009) and the dearth of novel antibiotics. Of the type 1 lantibiotics, nisin, mutacin and planosporicin have been shown to be active against multi-drug resistant gram-positive pathogens (Severina et al., 1998, Parrot et al., 1989, Castiglione et al., 2007), Pep5 and epidermin inhibit *Staphylococcus epidermidis* adhesion to catheters (Fontana et

al., 2006) and epidermin and gallidermin are active against *Propionibacterium acnes* (Kellner et al., 1988). Other lantibiotics, or their producer strains, have been used as food preservatives and as oral and gastrointestinal antimicrobials/probiotics (Cleveland et al., 2001, Wescombe et al., 2006, Wescombe et al., 2009). As a consequence of this increased interest in lantibiotics, a concerted effort has taken place to identify new and improved forms of these peptides. Culture-based screening strategies have in the past been responsible for the identification of lantibiotics produced by bacteria isolated from diverse microbial niches including the oral cavity, intestine, soil, kefir grains and milk (Dabard et al., 2001, Castiglione et al., 2007, Ryan et al., 1996, Beasley and Saris, 2004, Hillman et al., 1998). Recently, an alternative means of identifying novel lantibiotics has emerged as a consequence of the increasing generation and availability of genomic and metagenomic sequence data. The availability of such information has recently led to the identification of the type 1 epidermin-like lantibiotic, Bsa (Daly et al., 2010) as well as type 2 lantibiotics such as haloduracin (McClerren et al., 2006, Lawton et al., 2007), lichenicidin (Begley et al., 2009, Dischinger et al., 2009), as well as a range of cyanobacteria-associated lantipeptides (Li et al., 2010). This has prompted the development of on-line tools and repositories such as BAGEL and BACTIBASE to facilitate such screening strategies (de Jong et al., 2006, de Jong et al., 2010, Hammami et al., 2007, Hammami et al., 2010). Notably, although an *in-silico* screen for *lanM* genes has recently resulted in the identification of 61 novel type 2 lantibiotic-like gene clusters (Begley et al., 2009), a corresponding screen for type 1 lantibiotics has not yet been described. Here we address this issue by

screening for clusters containing genes homologous to the nisin A biosynthetic genes *nisB* (representing *lanB*) and *nisC* (representing *lanC*). In each case, the regions flanking the newly identified *lanB*/*lanC* genes were subjected to further *in-silico* analysis to determine if they are potential lantibiotic/lantipeptide-associated gene clusters. This included a search of nearby open reading frames (orfs) which might encode a corresponding LanA, defined as being of relatively short length (approx 60 amino acids) and containing an uneven distribution of cysteine, threonine and serine amino acids within the propeptide region. Using this approach, 27 novel type 1 lantibiotic/lantipeptide-encoding clusters were identified. Subsequent screening using the newly identified LanA, B and C homologs as driver sequences revealed a further 22 gene clusters, resulting in a total of 49 putative novel type 1 lantibiotic clusters. Significantly, many of these clusters are present in species, genera and phyla not previously associated with lantibiotic/lantipeptide production and are predicted to encode peptides which represent completely new type 1 subclasses.

3.3 MATERIALS AND METHODS

3.3.1 Screening of genomic databases

Using the nisin modification enzyme NisC (GenBank accession number CAA79470) as a driver sequence, all fully sequenced genomic sequences (approx. 1178 at time of study; Dec 2009) were mined for homologs using Genomic-BLAST (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). BLASTs were carried out with default parameters; criteria for homolog detection were a threshold of $1e^{-7}$ and greater than 20% identity.

3.3.2 Bioinformatic analysis of *lanC*-containing gene clusters

In cases where novel *lanC*-like genes encoding enzymes were identified, the arrangement of adjacent genes was visualised using the genome viewer on NCBI, and individual orfs were subjected to BLAST analysis to identify those potentially involved in lantibiotic production or immunity. The predicted LanA, LanB and LanC proteins from these operons were each in turn used for further *in-silico* screens to determine their similarities to corresponding proteins associated with known lantibiotics and to identify additional novel clusters. In instances where a LanC- and LanB-, but not a LanA-, encoding gene were annotated, intergenic regions were inspected following translation by the Seqbuilder program of the DNASTAR Lasergene 8 software package to investigate the presence of potentially unidentified *lanA* genes. The tblastn program was then used to search all sequenced DNA for related peptides.

3.2.3 Phylogenetic analysis

Protein alignments were generated by MUSCLE (Edgar, 2004). Sequence alignment were viewed and edited for publication with Jalview alignment editor (Waterhouse et al., 2009). These alignments were used to establish phylogenetic trees in Phylip (Felsenstein, 1989) which were subsequently visualised using the Dendroscope package (Huson et al., 2007).

3.4 RESULTS AND DISCUSSION

3.4.1 *In-silico* screen for *lanC* genes

An *in-silico* screen for LanC homologues, using the NisC sequence as a driver, resulted in the identification of 56 homologues. Of these 7 have previously been associated with lantibiotic production, 11 were orphan homologs (in that no other lantibiotic-associated genes were identified in close association) (**Table 1**), 9 were encoded within a cluster in which no *lanA* could be detected (**Table 2**) and one cluster contained two LanCs (but no structural peptide). The remaining 27 potential lantibiotic/lantipeptide-encoding gene clusters all contained putative *lanA*, *B* and *C* genes (**Table 3**). The genes flanking the 27 novel *lanC*-like genes were subjected to further bioinformatic analysis to determine the presence of other orfs that share homology with genes linked to lantibiotic production or immunity. While these 27 clusters are the primary focus of this *in-silico* analysis, the sequences of the newly identified LanA, B and C proteins associated with these clusters were in turn used for further *in-silico* screens. This approach uncovered an additional 22 clusters (**Table 4**) that were also predicted to be novel lantibiotic/lantipeptide-encoding clusters, thereby yielding a total of 49 novel type 1 clusters.

All except one of the 27 gene clusters revealed following the initial screen were located within the genomes of *Firmicutes* and *Actinobacteria*. The exception was *Chitinophaga pinensis* DSM 2588 of the phylum *Bacteroidetes*. Of the other 26, the genera most commonly associated with lantibiotic production were *Bacillus*, *Geobacillus*, *Clostridium*, *Enterococcus*, *Streptococcus*, *Frankia* and *Streptomyces*. In many cases the novel clusters associated with a specific

Table 1. A selection of bacterial genomes in which isolated genes encoding LanA, LanB or LanC homologs were identified.

Clusterless Homologs	Accession No.	LanA only	LanB only	LanC only
Anoxybacillus flavithermus WK1	NC_011567		Aflv_2440	
Bacillus sp. B14905	NZ_AAXV00000000		BB14905_21668	
Paenibacillus larvae subsp. *larvae* BRL-230010	NZ_AARF00000000			Plarl_010100024193
Lactobacillus crispatus MV-1A-US	NZ_ACOG00000000			HMPREF0507_00422
Lactobacillus crispatus JV-V01	NZ_ACKR00000000			HMPREF0506_0642
Haliangium ochraceum DSM 14365	NC_013440			Hoch_3102
Haliangium ochraceum DSM 14365	NC_013440			Hoch_4144
Haliangium ochraceum DSM 14365	NC_013440		Hoch_0066	
Pedobacter sp. BAL39	NZ_ABCM00000000		PBAL39_02527	
Peptoniphilus lacrimalis 315-B	NZ_ADDO00000000		HMPREF0628_0526	
Peptoniphilus lacrimalis 315-B	NZ_ADDO00000000		HMPREF0628_0527	
Lactococcus lactis subsp. *lactis* KF147	NC_013656			
Frankia alni ACN14a	NC_008278		FRAAL2701	
Frankia sp. CcI3	NC_007777			Francci3_0205
Frankia sp. CcI3	NC_007777			Francci3_3997
Peptoniphilus lacrimalis 315-B	NZ_ADDO00000000		HMPREF0628_0527	
Bifidobacterium longum subsp. *infantis* ATCC 15697	NC_011593			
Streptomyces sp. AA4	NZ_ACEV00000000			
Streptococcus pneumoniae CGSP14	NC_010582			
Saccharopolyspora erythraea NRRL 2338	NZ_ABFV00000000			SACE_4959
Kordia algicida OT-1	NZ_ABIB00000000			KAOT1_07113
Streptococcus dysgalactiae GGS_124	NC_012891			SDEG_0295
Microscilla marina ATCC 23134	NZ_AAWS00000000		M23134_07394	
Microscilla marina ATCC 23134	NZ_AAWS00000000		M23134_05752	
Microscilla marina ATCC 23134	NZ_AAWS00000000		M23134_07275	
Microscilla marina ATCC 23134	NZ_AAWS00000000		M23134_01545	
Microscilla marina ATCC 23134	NZ_AAWS00000000			M23134_07404
Streptomyces sp. Mg1	NZ_ABJF00000000		SSAG_05771	
Frankia sp. EuI1c	NZ_ADDL00000000	FraEuI1cDRAFT_6351		
Geobacillus sp. Y412MC52	NZ_ACNM00000000	GYMC52DRAFT_3129		
Geobacillus sp. Y412MC61	NC_013411	GYMC61_1158		
Streptococcus pyogenes M1 GAS	NC_002737	SPy_1083		
Streptomyces griseus subsp. *griseus* NBRC 13350	NC_010572			SGR_6574
Clostridium kluyveri DSM 555	NC_009706			CKL_3505
Spirosoma linguale DSM 74	NC_013730		Slin_0903	
Spirosoma linguale DSM 74	NC_013730		Slin_2131	

Table 2. Gene clusters encoding LanB and LanC, but not LanA, homologs

Species (Cluster No.)	Accession No.	LanB	LanC
Frankia alni ACN14a I	NC_008278	FRAAL2701	FRAAL2700
Frankia sp. CcI3 IV	NC_007777	Francci3_2033	Francci3_2032
Frankia EAN1pec II	NC_009921	Franean1_2799	Franean1_2800
Frankia sp. EuI1c II	NZ_ADDL00000000	FraEuI1cDRAFT_6786	FraEuI1cDRAFT_6785
Bacillus clausii KSM-K16	NC_006582	ABC3559	ABC3558
Clostridium cellulovorans 743B	NZ_ACPD00000000	ClocelDRAFT_0447	ClocelDRAFT_0445 / _0452
Bacillus cereus AH1273	NZ_ACMT00000000	bcere0030_58380	bcere0030_58400
Bacillus thuringiensis serovar berliner ATCC 10792	NZ_ACNF00000000	bthur0008_53920	bthur0008_53930
Bacillus thuringiensis IBL 200	NZ_ACNK00000000	bthur0013_59170	bthur0013_59180
Streptococcus pyogenes MGAS9429	NC_008021	MGAS9429_Spy0926	MGAS9429_Spy0924
Catenulispora acidiphila DSM 44928	NC_013131	Mentioned; Caci_4205	Caci_4204
Frankia sp Cc13 V	NC_007777	Francci3_3530	Francci3_3531
Microscilla marina ATCC 23134	NZ_AAWS00000000	M23134_05752	M23134_05756
Staphylococcus capitis SK14	NZ_ACFR00000000	STACA0001_2327	STACA0001_2326
Streptomyces sp. Mg1 I	NZ_ABJF00000000	SSAG_03540	SSAG_03541

Table 3. Bacterial genomes in which 27 uncharacterised type 1 lantibiotic clusters were identified following a NisC-led *in-silico* screen

Species (Cluster No.)	Accession No.
Frankia alni ACN14a (II)	NC_008278
Frankia sp Cc13 (I)	NC_007777
Frankia sp Cc13 (II)	NC_007777
Frankia sp Cc13 (III)	NC_007777
Frankia EAN1pec (I)	NC_009921
Frankia EAN1pec (III)	NC_009921
Frankia sp. EuI1c (I)	NZ_ADDL00000000
Salinispora arenicola CNS-205*	NC_009953
Stackebrandtia nassauensis DSM 44728 (I)	NC_013947
Stackebrandtia nassauensis DSM 44728 (II)	NC_013947
Streptomyces clavuligerus ATCC 27064 (I)	NZ_ADGD00000000
Streptomyces clavuligerus ATCC 27064 (II)	NZ_ADGD00000000
Streptomyces coelicolor A3(2) (I)	NC_003888
Streptomyces coelicolor A3(2) (II)	NC_003888
Streptomyces sp. Mg1 (II)	NZ_ABJF00000000
Streptomyces griseoflavus Tu4000 (I)	NZ_ACFA00000000
Streptomyces griseoflavus Tu4000 (II)	NZ_ACFA00000000
Streptomyces griseoflavus Tu4000 (III)	NZ_ACFA00000000
Bacillus cereus F65185	NZ_ACMO00000000
Bacillus mycoides DSM 2048	NZ_ACMU00000000
Clostridium perfringens CPE str. F4969	NZ_ABDX00000000
Enterococcus faecalis Fly1	NZ_ACAR00000000
Geobacillus kaustophilus HTA426	NC_006510
Geobacillus thermodenitrificans NG80-2	NC_009328
Geobacillus sp. G11MC16	NZ_ABVH00000000
Streptococcus thermophilus LMG 18311*	NC_006448
Chitinophaga pinensis DSM 2588 I	NC_013132

*The existence of a lantibiotic gene cluster within these strains has been referred to briefly, (Penn et al., 2009) and (Bolotin et al., 2004, Liu et al., 2009) respectively, but these clusters have not been the focus of a detailed bioinformatic analysis.

Table 4. Bacterial genomes in which 22 additional type 1 lantibiotic gene clusters were identified following an *in-silico* screen using the LanA, B, and C homologs, corresponding to the clusters referred to in **Table 3**, as leader sequences.

Species (Cluster No.)	Accession No.
Thermomonospora curvata DSM 43183	NC_013510
Frankia EAN1pec (IV)	NC_009921
Streptomyces viridochromogenes DSM 40736	NZ_ACEZ00000000
Streptomyces sp. SPB74	NZ_ABJG00000000
Streptomyces lividans TK24	NZ_ACEY00000000
Catenulispora acidiphila DSM 44928	NC_013131
Streptomyces sp. Mg1 (III)	NZ_ABJF00000000
Nocardiopsis dassonvillei subsp. *dassonvillei* DSM 43111	NZ_ABUI00000000
Micromonospora aurantiaca ATCC 27029 (I)	NZ_ADBZ00000000
Micromonospora aurantiaca ATCC 27029 (II)	NZ_ADBZ00000000
Bacillus cereus AH1272	NZ_ACMS00000000
Staphylococcus aureus subsp. *aureus* D139	NZ_ACSR00000000
Staphylococcus aureus subsp. *aureus* H19	NZ_ACSS00000000
Actinomyces sp. oral taxon 848	NZ_ACUY00000000
Parachlamydia acanthamoebae str. Hall's coccus	NZ_ACZE00000000
Corynebacterium lipophiloflavum DSM 44291	NZ_ACHJ00000000
Staphylococcus aureus A9765	NZ_ACSN00000000
Chitinophaga pinensis DSM 2588 (II)	NC_013132
Spirosoma linguale DSM 74	NC_013730
Pedobacter heparinus DSM 2366	NC_013061
Kordia algicida OT-1	NZ_ABIB00000000
Microscilla marina ATCC 23134	NZ_AAWS00000000

genus, such as those found on the *Streptomyces* and *Frankia* genomes, showed at least some similarity to each other. It was also noted that several of the genomes in which a cluster was located also contained an additional cluster(s) (**Table 3**), or other genes predicted to encode additional LanA, B or C proteins (**Table 2**), elsewhere in the genome. The 27 clusters are described below and are grouped according to the phylum and genus of the associated strain.

3.4.2 Type 1 lantibiotic gene clusters in *Actinobacteria*

3.4.2.1 *Identification of novel Frankia-associated lantibiotic gene clusters*

The *Frankia* are nitrogen-fixing, root nodule-forming filamentous *Actinobacteria* that live in symbiosis with actinorhizal plants. All species of *Frankia* are closely related (Normand et al., 2007). To date, four *Frankia* genomes have been sequenced, i.e. *Frankia alni* ACN14a, *Frankia* sp. EAN1pec, *Frankia* sp. Cc13 and *Frankia* sp. EUI1c, and although no *Frankia*-associated bacteriocins have previously been reported, a number of predicted lantibiotic clusters can be found in each case (**Figure 1**) in addition to a number of apparently LanB- and LanC-encoding genes which do not have an accompanying *lanA* (**Table 2**). This latter phenomenon could be a result of the frequent rearrangements which occur in *Frankia* strains (Normand et al., 2007). Of the clusters identified, many resemble clusters associated with another genus of *Actinobacteria*, the *Streptomyces*.

<u>*Frankia alni* ACN14a:</u> The genome sequence of *F. alni* ACN14a (Normand et al., 2007) contains one complete cluster, *F. alni* ACN14a II which includes the

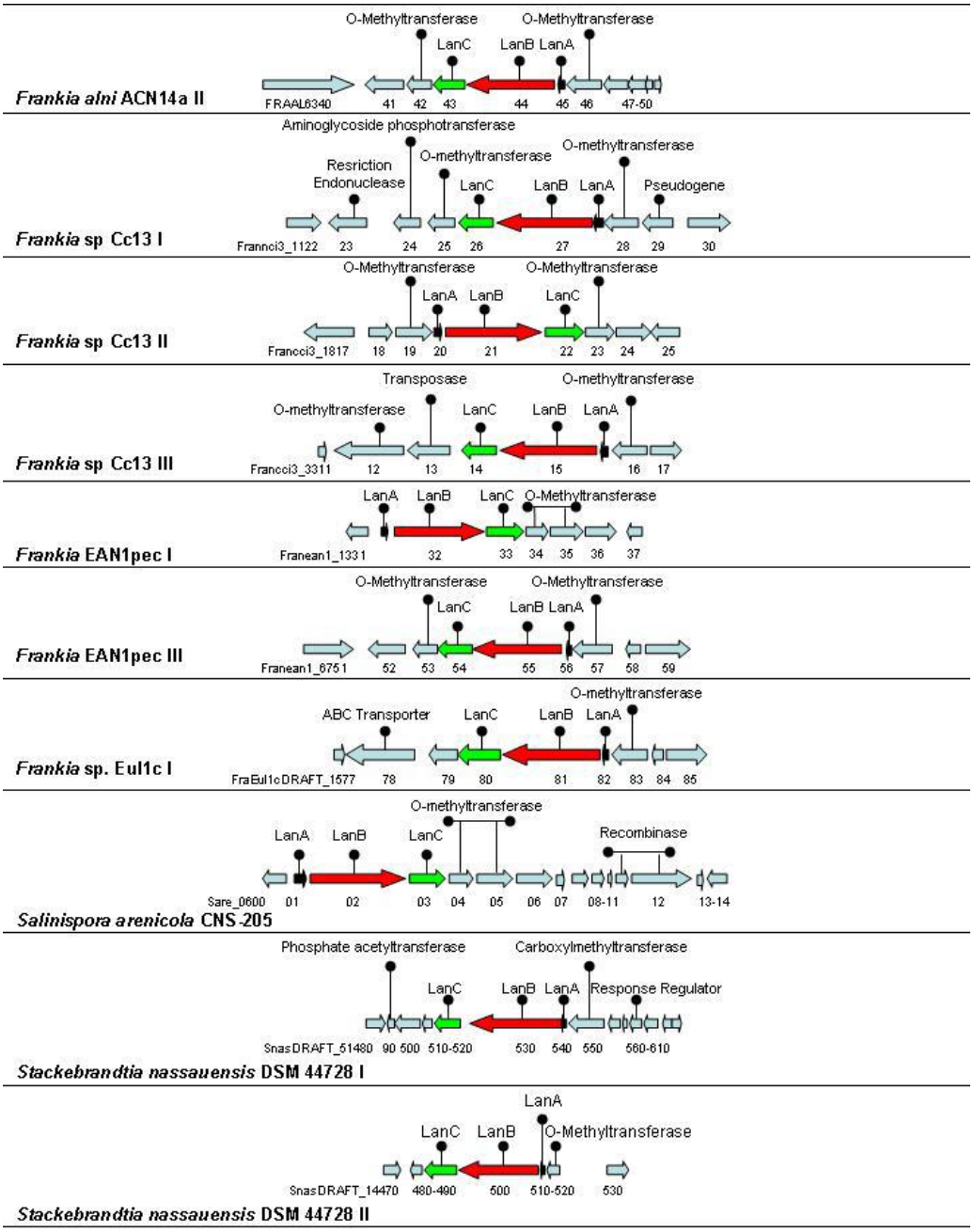

Figure 1

Diagrammatic representation of the non-*Streptomyces Actinobacteria* type 1 lantibiotic operons, identified in the first round of screening, which contain genes predicted to encode a structural peptide LanA, and the modification enzymes LanB and LanC. Structural peptides are coloured in black, dehydratases in red, synthetases in green and other ORFs in blue.

predicted LanA prepropeptide, FRAAL6345, FRAAL6344 and FRAAL6343 (encoding a putative LanB and LanC, respectively). The LanA peptide does not resemble any of the previously characterised type 1 lantibiotic propeptides but is 79% identical to Franean1_0057 of *Frankia* sp. EAN1pec (see below). The LanB and LanC proteins resemble those associated with other *Frankia*, as well as *Streptomyces*, clusters. The LanC protein is also 62% identical to Tcur_4648 of *Thermomonospora curvata* DSM 43183 (NC_013510), which itself appears to be encoded by an orf within a novel lantibiotic gene cluster. The ACN14a II cluster is also predicted to encode two proteins which share homology with O-methyltransferases (FRAAL6342 and FRAAL6346). O-methyltransferases contribute to the production of a number of non-ribosomal antibiotics (Li et al., 2009, Tsukada et al., 2010) and catalyse the methylation of hydroxyl group(s) on deoxysugar rings to protect the reactive hydroxyl group from undesired modifications and can alter the solubility and pharmacokinetic properties of the resulting molecule (Zubieta et al., 2001). Although O-methyltransferases have not previously been associated with lantibiotic production, this study reveals that many *Actinobacteria*-associated type 1 clusters possess genes predicted to encode these enzymes.

<u>*Frankia* sp. Cc13:</u> The *Frankia* sp. Cc13 genome (Normand et al., 2007) contains three gene clusters of interest (*Frankia* sp. Cc13 I, *Frankia* sp. Cc13 II and *Frankia* sp. Cc13 III). Two O-methyltransferase-encoding genes were identified in each case and it was noted that the associated LanB and LanC proteins are similar to one another and to numerous others predicted to be produced by

Frankia and *Streptomyces* species. In contrast, there is a lack of homology between the three *lanA* genes. The *lanA* gene from cluster I was not previously annotated and was only identified following closer inspection of the DNA sequence. The cluster II-associated LanA, Francci3_1820, most closely resembles *Frankia* sp. EuI1c FraEuI1cDRAFT_6351 (69% identity) while the third, and also previously unannotated LanA appears to be one of an extended group of *Frankia*- and *Streptomyces*-associated LanAs that includes *Frankia* sp. EuI1c FraEuI1cDRAFT_1582 (56% identity).

Frankia EAN1pec: The *Frankia* EAN1pec genome (Normand et al., 2007) contains 4 putative LanB-encoding genes three of which correspond to potential lantibiotic/lantipeptide-associated gene clusters (*Frankia* EAN1pec I, *Frankia* EAN1pec III and *Frankia* EAN1pec IV) which again resemble those of *Streptomyces* and other *Frankia* species, and contain O-methyltransferase-encoding genes. Within the first cluster, a putative LanA prepropeptide, encoded by a previously unannotated orf located between Franean1_1331 and the LanB determinant, is homologous to a number of other LanAs, including Sare_0601 of *Salinispora arenicola* CNS-205 (55% identity). The cluster is also noteworthy be virtue of the presence of two LanC-encoding genes, Franean1_1333 and Franean1_1336. Within *Frankia* EAN1pec III, the LanA peptide, encoded by Franean1_6756 is 42% identical to FraEuI1cDRAFT_6351 of *Frankia* sp. EuI1c while finally, a screen using *F. alni* ACN14a FRAAL6345 as a driver led to the identification of yet another cluster (consisting of at least Franean1_0057-0055) which closely resembles cluster II of *F. alni* ACN14a II.

Frankia sp. EuI1c: *Frankia* sp. EuI1c contains a single putative lantibiotic/lantipeptide gene cluster (*Frankia* sp. EuI1c I) which again contains LanB, C and O-methyltransferase genes typical of *Frankia* and *Streptomyces* clusters. The associated LanA homolog (FraEuI1cDRAFT_1582) is notable by virtue of being 46% identical to SSCG_03316, a known LanA of *Streptomyces clavuligerus* ATCC 27064 while a gene encoding an ABC transporter related protein (FraEuI1cDRAFT_1578) is also present.

3.4.2.2 *Identification of novel Salinispora -associated lantibiotic gene clusters*

Salinispora are marine *Actinobacteria*. There are two recognised species, *S. tropicalis* and *S. arenicola.* Representatives have been sequenced in each case and genes predicted to encode non-lantibiotic bacteriocins have been identified in both cases (Penn et al., 2009). The existence of a putative lantibiotic/lantipeptide cluster, between Sare_0602 and Sare_0623, in the genome of *S. arenicola* CNS-205 was noted previously (Penn et al., 2009). However, this cluster has not been the subject of a detailed bioinformatic characterisation. Our analysis reveals that Sare_0601 apparently encodes a LanA peptide which is 88% identical to that encoded by MicauDRAFT_5818 of *Micromonospora aurantiaca* ATCC 27029. The proteins encoded by Sare_0602 (LanB) and Sare_0603 (LanC) also resemble other ATCC 27029-associated proteins (encoded by MicauDRAFT_5819 (71% identity) and MicauDRAFT_5820 (75% identity)), thereby revealing an additional novel cluster in

Micromonospora, a genus better known for its production of non-ribosomal antibiotics such gentamycin and netamycin (Berdy, 2005) (**Table 4**).

3.4.2.3 *Identification of novel Stackebrandtia-associated lantibiotic gene clusters*

Stackebrandtia are aerobic, non-motile *Actinobacteria* which have been isolated from soil (Labeda and Kroppenstedt, 2005). There are only 2 associated species i.e. *S. albiflava* and *S. nassauensis* and *in-silico* analysis of *S. nassauensis* DSM 44728 (NC_013947) reveals the presence of two similar clusters (*S. nassauensis* DSM 44728 I and *S. nassauensis* DSM 44728 II) (**Figure 1**). The hypothetical LanA, encoded by Snas_5416, of the first cluster showed a singular homology of 78% identity to Snas_3601 of the second cluster. The corresponding LanBs (Snas_5417 and Snas_3602) are 62% identical while the LanCs (Snas_5418 and Snas_3603) are 68% identical.

3.4.2.4 *Identification of novel Streptomyces-associated lantibiotic gene clusters*

Bacteria from the genus *Streptomyces*, comprising over 500 species, are filamentous, high G-C bacteria found frequently in soil and rotting vegetation. They are the most numerous and ubiquitous soil bacteria (Hodgson, 2000). *Streptomyces* are also responsible for the production of over two-thirds of the clinically useful antibiotics of natural origin (e.g., neomycin, chloramphenicol) (Watve et al., 2001). Although a number of *Streptomyces*-associated bacteriocins, such as ancovenin (Kido et al., 1983) and cinnamycin (Widdick et

al., 2003), have been identified, this number is relatively small considering the size of the genus. As was apparent above, our *in-silico* analysis has revealed that many *Streptomyces* possess potentially lantibiotic-encoding gene clusters which resemble those found in *Frankia*. Once again, the majority of these clusters contain O-methyltransferases (**Figure 2**).

Streptomyces clavuligerus ATCC 27064: *S. clavuligerus* is an aerobic, mesophillic *Streptomyces* sp.. While there have been no previous reports of bacteriocin production by this species, two lantibiotic clusters were found to be present on the genome of *S. clavuligerus* ATCC 27064. In the first of these clusters, the associated hypothetical LanA, B and C proteins (SSCG_01498-01496) are 63%, 42% and 50% identical to the corresponding proteins of *Frankia* sp. CcI3 II. BLAST analysis of these proteins also revealed another novel cluster in *Streptomyces viridochromogenes* DSM 40736 corresponding to SvirD4_23440 (LanA; 50% identity), SvirD4_23449 (LanB; 36% identity) and SvirD4_23454 (LanB; 45% identity) (**Table 4**). The second *S. clavuligerus* cluster, which contains SSCG_03316 (LanA), SSCG_03317 (LanB) and SSCG_03318 (LanC), resembled clusters present in a number of other strains such as that of *Streptomyces griseus* subsp. *griseus* NBRC 13350 (Ohnishi et al., 2008) (73%, 56% and 64% identity, respectively). BLAST analysis of these sequences also led to the identification of yet another novel cluster in *Streptomyces* sp. SPB74 (SSBG_01041 [LanA] 69% identity and SSBG_01042 [LanB] 58% identity).

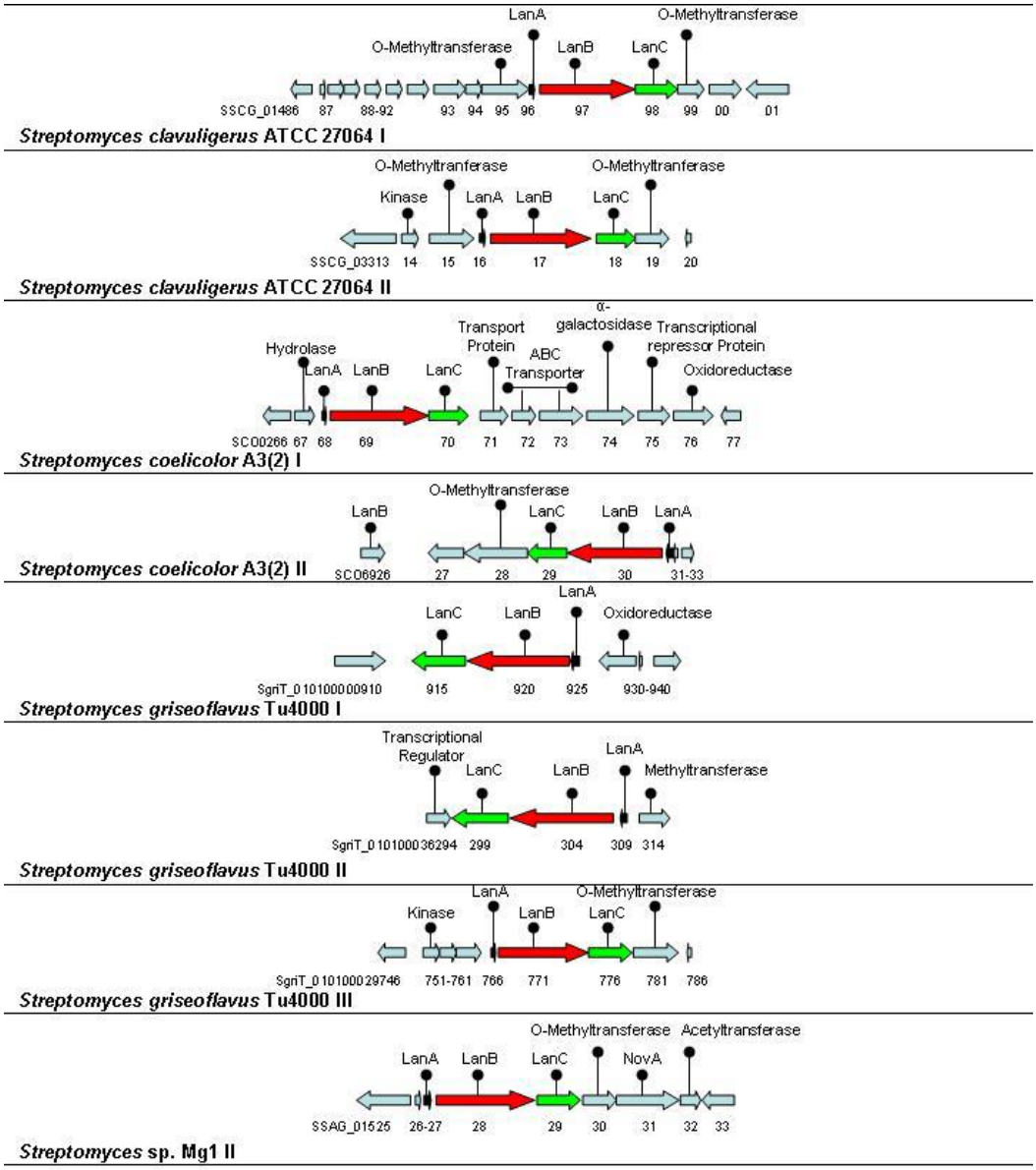

Figure 2

Diagrammatic representation of the *Streptomyces* type 1 lantibiotic operons, found in the original NisC screen, which contain genes predicted to encode a structural peptide LanA, and the modification enzymes LanB and LanC. Structural peptides are coloured in black, dehydratases in red, synthetases in green and other ORFs in blue.

Streptomyces coelicolor A3(2): *Streptomyces coelicolor* A3(2) (NC_003888) is the best characterised representive of its genus (Hopwood, 1999) and was the first *Streptomyces* strain to have its genome sequenced (Bentley et al., 2002). Although bacteriocins/bacteriocin-like peptides are known to be produced by this species (e.g. the class III morphogenic peptide SapB (Kodani et al., 2004)), such peptides have not previously been associated with this strain. Here BLAST analysis revealed the presence of two lantibiotic/lantipeptide clusters (*S. coelicolor* A3(2) I and *S. coelicolor* A3(2) II). The first of these clusters, containing SCO0268 (LanA), SCO0269 (LanB) and SCO0270 (LanC), very closely resembles *Streptomyces griseoflavus* Tu4000 cluster II (see below). Subsequent BLAST searches with the A3(2) cluster I-associated proteins led in turn to the discovery of an almost identical cluster in *Streptomyces lividans* TK24 which contains SSPG_07329 (LanA; 100% identity), SSPG_07328 (LanB; 99% identity) and SSPG_07327 (LanC; 100% identity) (**Table 4**). The second cluster, *Streptomyces coelicolor* A3(2) II, is predicted to encode two LanA peptides, (SCO6932 [43aa] and SCO6931 [59aa]) which are 97% identical to each other, as well as LanB (SCO6930) and LanC (SCO6929) proteins with homology (39-46% identity) with corresponding proteins associated with *Frankia* sp. CcI3. Such analysis also revealed another cluster of interest in the actinomycete, *Catenulispora acidiphila* DSM 44928 (NC_013131; [**Table 4**]).

Streptomyces griseoflavus Tu4000: Three lantibiotic/lantipeptide clusters were identified on the genome of this anaerobic, terrestrial *Streptomyces*. Although the LanA encoded within the first cluster (SgriT_010100000925) does not

significantly resemble any other protein, the associated LanB (SgriT_010100000920) and LanC (SgriT_010100000915) proteins are homologous to the corresponding proteins of *S. coelicolor* A3(2) cluster I and Tu4000 cluster II. BLAST searches using the cluster I proteins as drivers also resulted in the identification of several additional clusters in *Nocardiopsis dassonvillei* DSM 43111 (NdasDRAFT_3161 [LanB] 30% identity), *Streptomyces sp.* Mg1 (SSAG_05771 [LanB] 37% identity) and two clusters on the genome of *M. aurantiaca* ATCC 27029 (NZ_ADBZ00000000; MicauDRAFT_5820 and MicauDRAFT_3008 [both LanBs] 35% identity). In addition to the components of the second cluster referred to above, an associated LanA (SgriT_010100036309) was also noted. In addition to the Tu4000 I and A3(2) I clusters, this cluster is also highly identical to that of *S. lividans* TK24 (SSPG_07329 [LanA] 97% identity; SSPG_07328 [LanB] 87% identity and SSPG_07327 [LanC] 89% identity). The LanA associated with the final cluster (SgriT_010100029766) again bears no homology with any other known peptides whereas the LanB (SgriT_010100029771) and LanC (SgriT_010100029776) corresponded to those of *Frankia* sp. EAN1pec II (39% and 44% identity, respectively).

3.4.3 Type 1 lantibiotic gene clusters in *Firmicutes*

3.4.3.1 *Identification of novel Bacillus-associated lantibiotic gene clusters*

Bacillus is a large and diverse genus of rod-shaped, sporulating, facultative aerobes which contains some pathogenic species. A number of type 1 lantibiotics have previously been characterized in this genus (e.g. subtilin (Nishio et al., 1983) and ericin (Stein et al., 2002)). The NisC-driven screen highlighted

the presence of a type 1 lantibiotic cluster in the genomes of two *Bacillus* strains i.e. *Bacillus cereus* F65185 and *Bacillus mycoides* DSM 2048 (**Figure 3**). Bioinformatic analysis of these clusters revealed two further clusters in *B. cereus* ATCC 14579 and *B. cereus* AH1272.

Bacillus cereus F65185: *B. cereus* F65185 is a mesophilic bacterium sourced from a human wound containing one lantibiotic/lantipeptide cluster which is unusual in that 3 orfs separate the putative LanB and C genes and the two have a divergent orientation. The predicted LanA (bcere0025_48310) does not resemble any other known lantibiotic prepropeptides. The LanB homolog (bcere0025_48320) resembles a putative LanB associated with *Clostridium cellulovorans* 743B (ClocelDRAFT_0452, 30% identity) while the predicted LanC (bcere0025_48280) most closely resembles two further 743B proteins (ClocelDRAFT_0452, 34% identity and ClocelDRAFT_0446, 28% identity). However the 743B strain lacks an associated LanA. Further BLAST analysis with the F65185-associated LanB highlighted the presence of a related protein within thiocillin-encoding gene cluster in *B. cereus* ATCC 14579 (Brown et al., 2009).

Bacillus mycoides DSM 2048: *B. mycoides* is a non-motile, non-pathogenic, saprophytic *Bacillus*, strains of which have been investigated with a view to their application as biological pesticides. Although representatives of this species have been associated with bacteriocin production (Sharma and Gautam, 2008), there are no published reports of lantibiotic-producing *B. mycoides*. The DSM 2048 genome contains a lantibiotic/lantipeptide cluster that contains

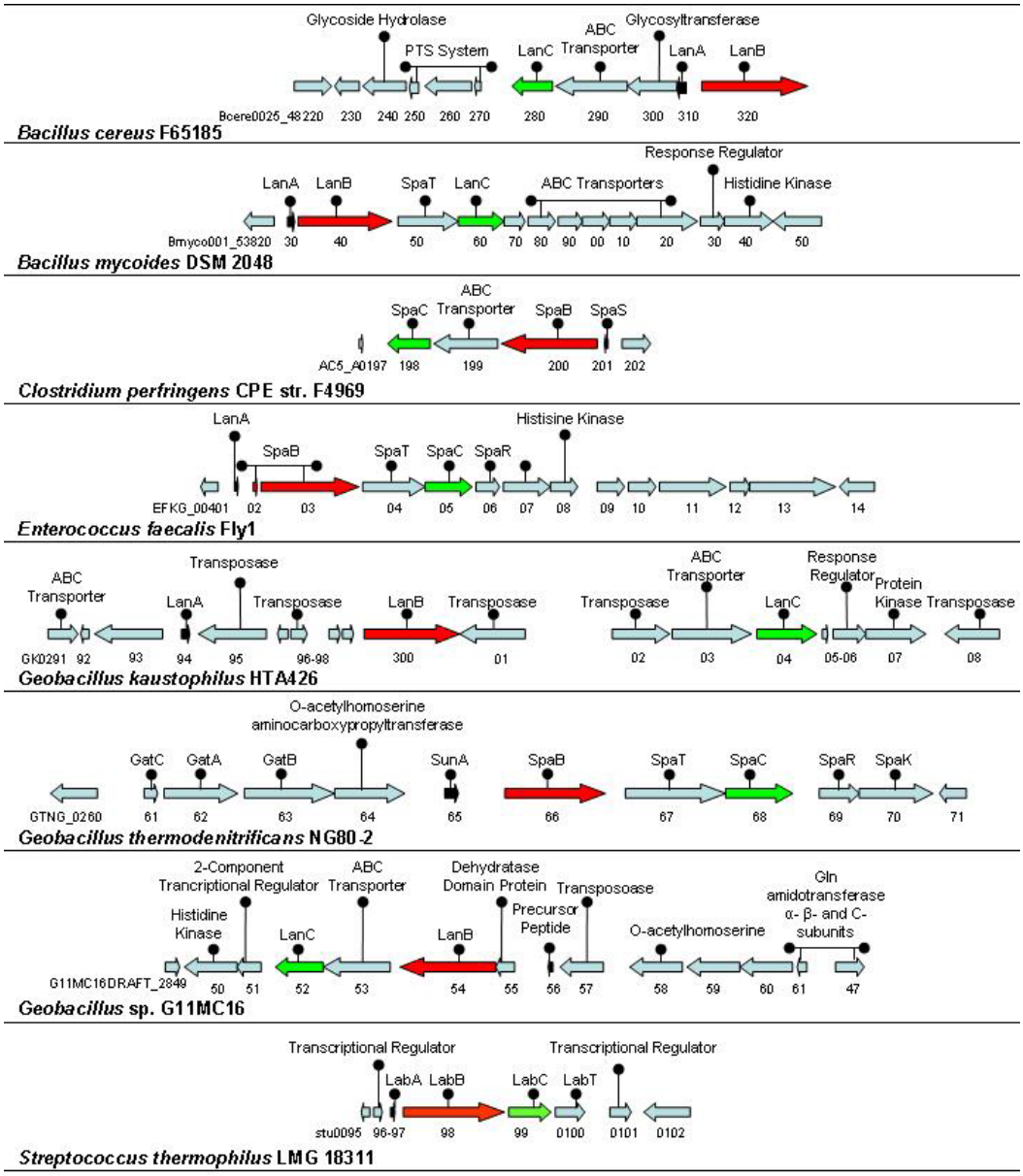

Figure 3

Diagrammatic representation of the *Firmicutes* type 1 lantibiotic operons, found in the original NisC screen, which contain genes predicted to encode a structural peptide LanA, and the modification enzymes LanB and LanC. Structural peptides are coloured in black, dehydratases in red, synthetases in green and other ORFs in blue.

putative *lanA*, *B*, *C* and other lantibiotic-associated genes which is very similar to other novel clusters in *B. cereus* AH1272 and *B. cereus* AH1273. Bmyco0001_53830 is the predicted prepropeptide and is 100% identical to the products of the *B. cereus* AH1272-associated bcere0029_28240 and bcere0029_28250. It is also 58% identical to prepropeptides associated with several *Staphylococcus*-associated Bsa lantibiotics, such as $BsaA2_{RF122}$ of *S. aureus* RF122 (Daly et al., 2010). It is thus apparent that the LanA is a member of the epidermin-like peptides. In addition to homologues in *B. cereus* AH1273 and AH1272, use of the DSM 2048-associated LanB and LanC sequences as drivers also surprisingly highlighted a gene cluster present in *Actinomyces* sp. oral taxon 848 (**Table 4**). In addition to homology with respect to LanB and LanC proteins (HMPREF0972_00932 and HMPREF0972_00933, respectively), the proposed LanA (HMPREF0972_00931; 86aa) is 43% identical to bmyco0001_53830 and bcere0029_28240 and bcere0029_28250 of *Bacillus mycoides* DSM 2048 and *Bacillus cereus* AH1272, respectively.

3.4.3.2 *Identification of novel Clostridium-associated lantibiotic gene clusters*

The *Clostridia* are gram postitive anaerobic, endospore-forming *Firmicutes* of which there are approximately 100 species. These include important pathogens such as *Clostridium difficile, Clostridium perfingens* and *Clostridium tetani*. Several *Clostridium*-associated bacteriocins have been characterised from this genus (Dineen et al., 2000, Li et al., 1982, Nieves and Castillo, 1982), but no type 1 lantibiotic producers have been identified to date. Here, bioinformatic

analysis revealed one *Clostridium*-associated lantibiotic/lantipeptide cluster, located on the genome of *C. perfringens* CPE str. F4969 (**Figure 3**).

C. perfringens CPE str. F4969: *C. perfringens* is one of the leading causes of food-borne illness in the developed world. It can also be responsible for wound and surgical infections (Narayan, 1982, Pearson et al., 1980). The predicted LanA (AC5_A0201) of strain F4969 is most closely related to the LanAs of *B. mycoides* DSM 2048 and *B. cereus* AH1272 (51% identity) while the proteins encoded by AC5_A0200 (LanB) and AC5_A0198 (LanC) most closely resemble proteins associated with *Geobacillus* sp. G11MC16 (G11MC16DRAFT_2954 and G11MC16DRAFT_2952; 34% and 35% identity respectively). Surprisingly, BLAST analysis also highlighted the presence of a related LanB homolog encoded within a lantibiotic/lantipeptide-like gene cluster in the genome of *Parachlamydia acanthamoebae* (phlylum *Chlamydiae*) str. Hall's coccus (pah_c028o031; 25% identity). Adjacent genes of note within the *P. acanthamoebae* cluster include pah_c028o029 (LanA) and pah_c028o030 (LanC).

3.4.3.3 *Identification of novel Enterococcus-associated lantibiotic gene clusters*

The enterococci are gram positive lactic acid bacteria which are common commensal organisms in the intestines of humans but can also be pathogens. Many *Enterococcus*-associated bacteriocins (enterocins) have been identified (Franz et al., 2007). Only one *Enterococcus*-associated lantibiotic, the type 2 peptide cytolysin, has been identified to date (Booth et al., 1996). Here we

describe genes which potentially encode the first type 1 *Enterococcus*-associated lantibiotic (**Figure 3**).

<u>*Enterococcus faecalis* Fly1:</u> *E. faecalis* Fly1 (NZ_ACAR00000000) is a non-motile, facultative anaerobe. Within its genome we identified a previously unannotated LanA determinant, through analysis of raw sequence data. The corresponding peptide is homologous to *C. perfringens* CPE str. F4969 (AC5_A0201; 68% identity), as well as a number of epidermin-like LanAs in other bacilli. The putative LanB protein is split across two orfs, EFKG_00402 (80 amino acids) and EFKG_0403 (942 amino acids), with both components most closely resembling the N-terminus of the dehydratase of *Streptococcus pyogenes* MGAS10270, MGAS10270_Spy0922. It is unclear whether the apparent frameshift in the Fly1 *lanB* is genuine or the result of a sequencing error. The LanC-like EFKG_00405, was most closely related to the corresponding protein in *G. thermodenitrificans* NG80-2 (SpaC GTNG_0268; 35% identity).

3.4.3.4 *Identification of novel Geobacillus-associated lantibiotic gene clusters*

Geobacilli are thermophillic (45-70^{o}C), aerobic, spore-forming *Firmicutes*. They have been isolated from various terrestrial and marine environments, in geothermal, temperate and permanently cold habitats. Reclassified in 2001 (Nazina et al., 2001), these bacteria are of industrial interest as sources of thermostable enzymes. Bacteriocins have been identified in *Geobacillus stearothermophilus* (Pokusaeva et al., 2009) and *Geobacillus thermoleovorans* (Novotny and Perry, 1992), and while screening for LanM-producing gene

clusters has highlighted the potential existence of a number of type 2 lantibiotics (Begley et al., 2009), associated type 1 lantibiotics have not previously been described. Here, three putative type 1 lantibiotic/lantipeptide-encoding clusters within the genomes of *Geobacillus kaustophilus* HTA426, *Geobacillus thermodenitrificans* NG80-2 and *Geobacillus sp.* G11MC16 (**Figure 3**) are described.

Geobacillus kaustophilus HTA426: *G. kaustophilus* grows optimally in aquatic environments at 60°C with an upper temperature limit of 74°C. From a lantibiotic persepective, genome sequencing of HTA426 revealed a hypothetical protein annotated as a 'lantibiotic precursor' GK0294. Our analysis revealed that this putative LanA is 91% identical to another prepropeptide encoded by the closely located GK0286 gene. It is also 100% identical to orphan 'lantibiotic precursor' homologs (GYMC52DRAFT_3129 and GYMC61_1158) in *Geobacillus* sp. Y412MC52 and *Geobacillus* sp. Y412MC61, respectively. More distantly related LanAs (79% identity) are also associated with the genomes of *Geobacillus thermodenitrificans* NG80-2 (GTNG_0265) and *Geobacillus* sp. G11MC16 (G11MC16DRAFT_2956). The homology between the *Geobacillus* LanAs is highest within the leader regions, but, as is the case with nisin-, epidermin- and streptin-like lantibiotics, a conserved serine and CTPGC motif in the N-terminus of the propeptide is present, which is believed to be involved in the binding of these lantibiotics to lipid II in the cell wall in gram positive bacteria (Hsu et al., 2004). BLAST analysis of the GK0286-encoded LanA highlighted the presence of another potential lantibiotic/lantipeptide cluster in

Corynebacterium lipophiloflavum DSM 44291 (57% identity with HMPREF0298_1795). Within the HTA426 cluster, the proteins predicted to be encoded by GK0300/301 (an apparently frameshifted *lanB*) and GK0304 are homologous to those associated with many other geobacilli. It was also noted that this cluster is less condensed than typical lantibiotic gene clusters in that there are insertions of 7, 5 and 3 genes (predicted to encode many transposases and small, hypothetical proteins) between the lantibiotic associated genes.

Geobacillus thermodenitrificans NG80-2 and *Geobacillus sp. G11MC16:* *G. thermodenitrificans* are facultative soil bacteria with denitrification qualities. Representatives of this species grow between 45°C and 73°C (optimum 65°C). NG80-2 was isolated from a deep-subsurface oil reservoir in Dagang oilfield, Northern China (Feng et al., 2007) and on the basis of *in-silico* analysis is potentially the producer of both a type 1 (see below) and type 2 lantibiotic (Begley et al., 2009). Our analysis reveals that the type I lantibiotic/lantipeptide operons in *G. thermodenitrificans* NG80-2 and *Geobacillus sp.* G11MC16 are very highly conserved. The two LanAs are 100% identical and the homology between these, and indeed the associated B and Cs, and the corresponding *G. kaustophilus* HTA426 proteins is discussed above. It was noted that the *lanB* of *Geobacillus sp. G11MC16* is apparently frameshifted (G11MC16DRAFT_2955 (176aa) and G11MC16DRAFT_2954 (848aa)) but that this is not the case in *G. thermodenitrificans* NG80-2 (GTNG_0266).

3.4.3.5 *Identification of novel Staphylococcus-associated lantibiotic gene clusters*

The staphylococci are non-sporeforming, non-motile *Firmicutes*. The genus *Staphylococcus* contains 33 species, most of which are harmless and reside normally on the skin and mucous membranes of humans and other organisms. However, staphylococci can also cause a wide variety of diseases either through toxin production or penetration and are a common cause of food poisoning and nosocomial infections. Several strains of *Staphylococcus epidermidis* have been shown to be producers of type 1 lantibiotics, including epidermin (Allgaier et al., 1985), Pep5 (Kaletta et al., 1989), epicidin 280 (Heidrich et al., 1998) and epilancin K7 (Vandekamp et al., 1995), gallidermin was isolated from *S. gallinarum* (Kellner et al., 1988) while Staphylococcin Au26 (Scott et al., 1992) and Bsa (Daly et al., 2010) were isolated from *S. aureus*. BLAST analysis has revealed that several other *S. aureus* strains possess gene clusters similar to those associated with Bsa and Bsa_{RF122} (Daly et al., 2010). These clusters were identified in *S. aureus* A9765, D139 and H19. In A9765, SAPG_01762 and SAPG_01760 correspond to the BsaA1 and BsaA2 peptides of *S. aureus* MW2 (97% and 100% identity, respectively). The precursor peptides of the D139 (SATG_00575 and SATG_00574; 76% identical to each other) and H19 (SAUG_01228 and SAUG_01229; 76% identical to each other) strains are 100% identical. The peptides encoded by SATG_00575 and SAUG_01229 are 93% identical to $BsaA1_{RF122}$ of *S. aureus* RF122 (93% identity) while those corresponding to SATG_00574 and SAUG_01228 are 100% identical to $BsaA2_{RF122}$.

3.4.3.6 *Identification of novel Streptococcus-associated lantibiotic gene clusters*

These facultative anaerobes of the phylum *Firmicutes* are spherical in shape and grow in long chains. Many species are part of the normal commensal flora of the mouth, skin, intestine and upper respiratory tract of humans but the genus also includes numerous human pathogens such as *Streptococcus pneumoniae*, *pyogenes* and *agalactiae*. The streptococci are known to be producers of type 1 lantibiotics (Geis et al., 1983, Nes et al., 2007), such as streptin (Wescombe and Tagg, 2003), some mutacins (MotaMeira et al., 1997, Hillman et al., 1998, Qi et al., 1999, Qi et al., 2000), nisin U and nisin U2 (Wirawan et al., 2006), as well as several non-lantibiotic bacteriocins. Here we discuss two clusters, identified in strains of *S. pyogenes* and *S. thermophilus* LMG 18311.

Streptococcus pyogenes MGAS10270: *S. pyogenes* (or Group A *Streptococcus*, GAS) is the cause of many important human diseases ranging from mild superficial skin infections to life-threatening systemic diseases. Bacteriocin production by these strains may give them a competitive advantage against the natural skin microbiota. It has previously been established that many *S. pyogenes* strains, as well as strains of *Streptococcus salivarius*, produce the type 2 lantibiotic salivaricin A or closely related variants (Wescombe et al., 2006). The type 1 streptins (1 and 2) and type 2 streptococcin A-FF22 are also *S. pyogenes* associated (Wescombe and Tagg, 2003, Hynes et al., 1993). Here our analysis focuses on a type 1 cluster within the genome of *S. pyogenes* MGAS10270 (Beres et al., 2006). This includes MGAS10270_Spy0919, which is 100% identical

to the propeptide sequence of streptin. While this lantibiotic is thus not novel, subsequent BLAST searches were revealing in that they highlighted the presence of a LanA with 97% identity in *S. pyogenes* MGAS10750 (MGAS10750_Spy0955) which is contained within a cluster which also encodes a LanB (MGAS10750_Spy0958) and LanC (MGAS10750_Spy0957).

Streptococcus thermophilus LMG 18311: *S. thermophilus* is a thermophillic, non-pathogenic *Streptococcus*. It is of major importance to the fermented dairy food industry. A number of non-lantibiotic bacteriocins (thermophilins) from this species have been characterised, including thermophilin 347 (Villani et al., 1995), thermophilin A (Ward and Somkuti, 1995) and thermophilin ST-1 (Aktypis and Kalantzopoulos, 2003). Strain LMG 18311 was sequenced in 2004 and at the time it was noted that bacteriocin production was one of the characteristics that distinguishes it from strain CNRZ1066 (Bolotin et al., 2004). While the existence of a putative lantibiotic/lantipeptide gene cluster in LMG 18311 has been reported (Bolotin et al., 2004, Liu et al., 2009), this cluster (**Figure 3**) has not been the focus of a detailed *in-silico* analysis. The associated LanA, encoded by stu0097, is homologous with that predicted to be encoded by SPCG_0144 of *S. pneumoniae* CGSP14 (88% identical) which, on the basis of previous *in-silico* analysis, is also within a lantibiotic gene cluster (Ding et al., 2009). The LanB protein (Stu0098) is 73% identical to SPCG_0145 of *S. pneumoniae* CGSP14 and 97% identical to a truncated LanB associated with *S. thermophilus* CNRZ1066 (Bolotin et al., 2004).

3.4.4 Type 1 lantibiotic gene clusters in *Bacteroidetes*

The Bacteroidetes are a highly diverse phylum found in soil, seawater and the skin and intestines of animals. The Bacteroidales class, which includes the genus *Bacteroides*, are the best-studied of the phylum. Bacteroides comprises the most substantial portion of the human gastrointestinal tract (Qin et al., 2010) some of which are opportunistic pathogens (Nobles, 1973).

3.4.4.1 *Identification of novel Chitinophaga-associated lantibiotic gene clusters*

Chitinophaga are rod-shaped mesophiles of the phylum *Bacteroidetes* which are noted for their ability to degrade chitin (Sangkhobol and Skerman, 1981). There have been no reports to date of bacteriocin production by any of the 10 *Chitinophaga* species**.** *Chitinophaga pinensis* DSM 2588 (NC_013132) is unusual in that it appears to be a *Bacteroidetes* possessing genes encoding a type 1 lantibiotic (**Figure 4**). There are two predicted LanA peptides, corresponding to Cpin_1438 and Cpin_1437, which are 50% identical as a consequence of similar N-terminii. Adjacent orfs of note include Cpin_1435 and Cpin_1440, predicted to encode a β-lactamase and a 2-component transcriptional regulator of the LuxR family, respectively. BLAST analysis of the associated LanB and LanC proteins (Cpin_1436 and Cpin_1439 respectively) revealed another putative LanB (Cpin_3392; 36% identity) and LanC (Cpin_3397; 23% identity) encoded within the same genome. Within this second *C. pinensis*-associated cluster, Cpin_3393 possess a number of features which suggest that it may be a LanA-encoding gene. Interestingly, BLAST analysis of the Cpin_3397-encoded LanC

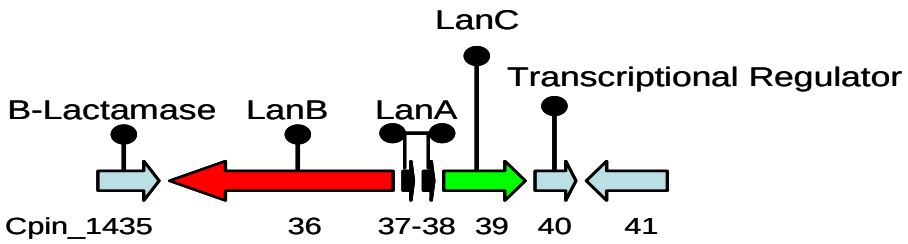

Figure 4

Diagrammatic representation of the *Chitinophaga pinensis* (*Bacteroidetes*) type 1 lantibiotic operons, found in the original NisC screen, which contain genes predicted to encode a structural peptide LanA, and the modification enzymes LanB and LanC. Structural peptides are coloured in black, dehydratases in red, synthetases in green and other ORFs in blue.

also led to the identification of a number of additional homologs apparently encoded within the genomes of strains not previously associated with lantibiotic production. The genome of another *Bacteroidetes*, *Spirosoma linguale* DSM 74, is notable in that it contains 4 putative LanB-encoding genes and 5 putative LanC-encoding genes. Of these only one LanB protein (Slin_4704; 31% identity) and one LanC protein (Slin_4705; 26% identity), are encoded within what appears to be a novel lantibiotic-associated gene cluster. This cluster contains 3 potentially LanA-encoding genes, Slin_4706-4708. Slin_4706 and Slin_4707, which are identical and share 58% identity with Slin_4708. The genome of another *Bacteroidetes* species, *Pedobacter heparinus* DSM 2366 (NC_013061), contains a cluster encoding two LanBs (Phep_0556 and Phep_0557; 37% and 36% identity, respectively), a LanC (Phep_0555; 33% identity) and a potential LanA (Phep_0553; no significant BLAST hits). A cluster within *Kordia algicida* OT-1 contains LanB (KAOT1_15523; 36% identity), LanC (KAOT1_15518; 30% identity) and LanA determinants (KAOT1_15533; no significant BLAST hits) while *Microscilla marina* ATCC 23134 potentially has five associated LanB homologs, but of these, the LanB corresponding to M23134_03921 (28% identity) was the only one to be located in close proximity to one of multiple LanC proteins (M23134_03925; 22% identity). The putative LanA, M23134_03926, does not resemble any other known proteins.

3.4.5 Phylogenetics of LanABC

The conserved nature of LanB and LanC proteins facilitated a phylogenetic analysis of their relatedness. The resultant cladogram of LanB enzymes (all

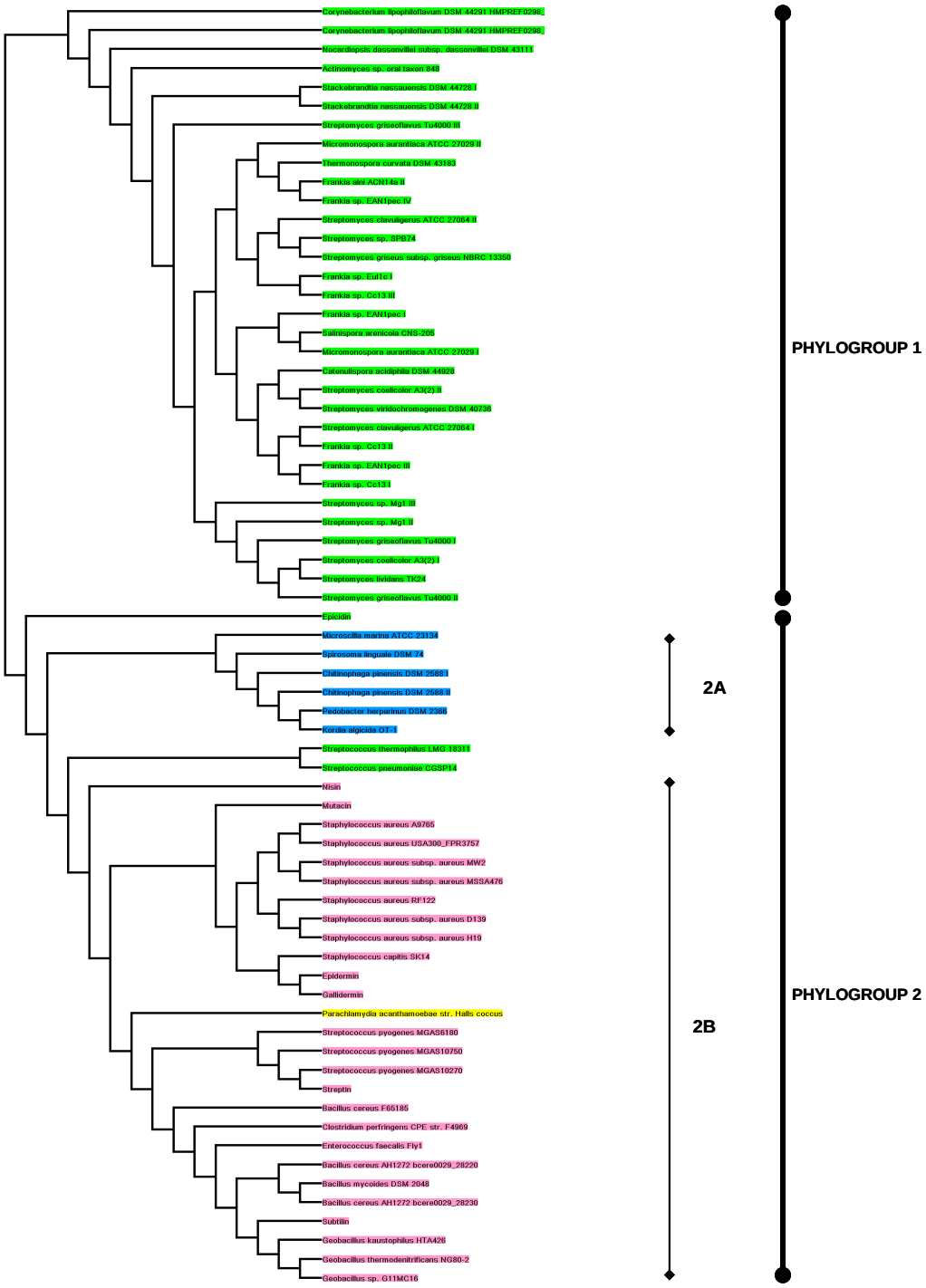

Figure 5

Cladogram of all the LanB enzymes from clusters encountered during the screen. Also included are dehydratases from some well-known lantibiotics

Green = *Actinobacteria*; Pink = *Firmicutes*; Blue = *Bacteroidetes*; Yellow = *Chlamydiae*

those identified in both screens, as well as a number of LanBs from previously analysed clusters) highlights the existence of two distinct phylogroups (**Figure 5**). The first phylogroup contains *Actinobacteria*-associated LanBs, all of which are from strains not previously known to be producers of lantibiotics/lantipeptides. The second contains a variety of lanthionine synthetases associated with known lantibiotics (nisin, subtilin, epidermin etc), some uncovered by previous *in-silico* analysis (e.g. *S. pneumoniae* CGSP14 (Ding et al., 2009)), novel clusters from genera with which lantibiotic production has previously been attributed, as well as genera not previously associated with lantibiotic production. Within this second phylogroup one finds two subgroups; one consisting of *Bacteroidetes*-associated LanBs (2A) and a second consisting of *Firmicutes*-associated LanBs (2B) as well as that from *P. ancanthamoeba*. Among the *Firmicutes*-associated LanBs further subclustering is evident. One common branch contains three offshoots; (i) *Bacillus*/*Geobacillus*/*Enterococcus*/*Clostridium*, (ii) *S. pyogenes* and (iii) *P. acanthamoebae* str. Hall's coccus LanBs. The *Staphylococcus* LanBs and that of mutacin 1140 (those associated with epidermin-like peptides) also form a distinct subgroup as do those encoded with the genomes of *S. thermophilus* LMG 18311 and *S. pneumoniae* CGSP14. Curiously the epicidin (*S. epidermidis*) LanB does not group with any other LanB.

The cladogram of the corresponding lanthionine synthetases (LanCs) is quite similar to that of the dehydratases (**Figure 6**). All can be positioned into one of two phylogroups (phylogroups 1 and 2). Phylogroup 1 contains six *Bacteroidetes*-associated LanC's. In contrast phylogroup 2 is large and can be

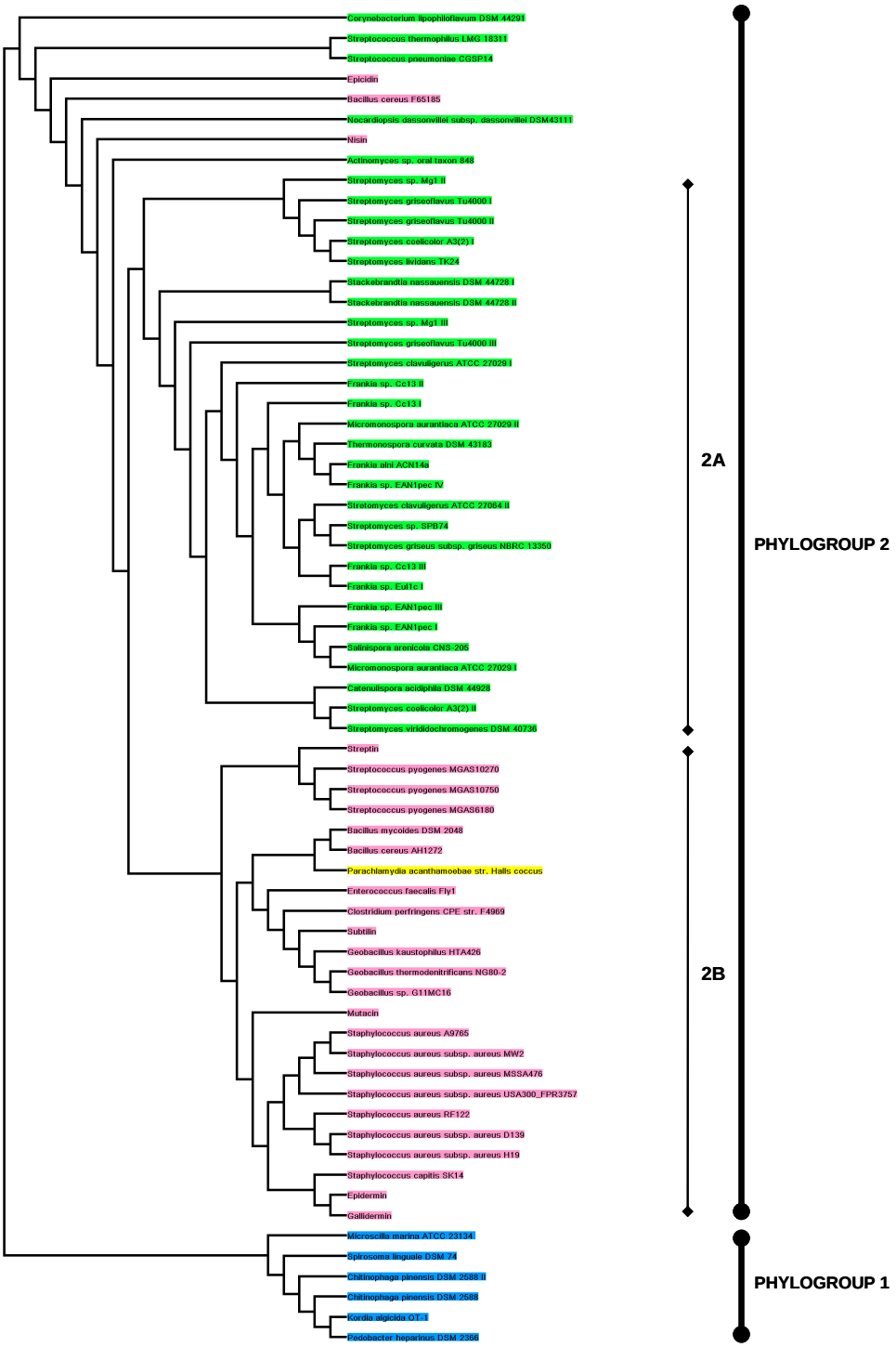

Figure 6

Cladogram of all the LanC enzymes from clusters encountered during the screen, as well as some from well-known lantibiotics

Green = *Actinobacteria*; Pink = *Firmicutes*; Blue = *Bacteroidetes*; Yellow = *Chlamydiae*

further divided into group 2A, which are *Actinobacteria*-associated, and the *Firmicutes*-associated group 2B. Further subgrouping follows the patterns identified from analysis of the LanB cladogram. 8 exceptional LanCs that avoid subgrouping include those associated with *C. lipophiloflavum* DSM 44291, *S. thermophilus* LMG 18311/*S. pneumoniae* CGSP14, epicidin, *B. cereus* F65185, *N. dassonvillei* subsp. *dassonvillei* DSM 43111, nisin-producing lactococci and *Actinomyces* sp. oral taxon 848.

A cladogram of the less highly conserved LanAs revealed 13 major branches, several of which contain only one corresponding LanA (**Figure 7**). Notably the various phylogroups do not group in a phylum specific manner to the same extent as was evident in LanB and LanC cladograms. The largest phylogroups, i.e. phylogroups 11 and 13, are those containing the nisin-like and epidermin-like peptides, respectively. While phylogroup 13 is, with the exception of the *Actinomyces* sp. oral taxon 848-associated LanA, composed of *Firmicutes*-associated LanAs, phylogroup 11 contains LanAs from both *Firmicutes* and *Actinobacteria*. This phylogroup contains three subgroups, with subgroup 11A containing LanAs from *E. faecalis* Fly1 and *C. perfringens* F4969, 11B contains *Actinobacteria*-associated LanAs and 11C contains both *Actionobacteria*- and *Firmicutes*-associated LanAs. Of the other phylogroups, phylogroups 6 and 7 are largest and contain *Chlamydiae*/*Bacteroidetes*- and *Actinobacteria*-associated LanAs, respectively.

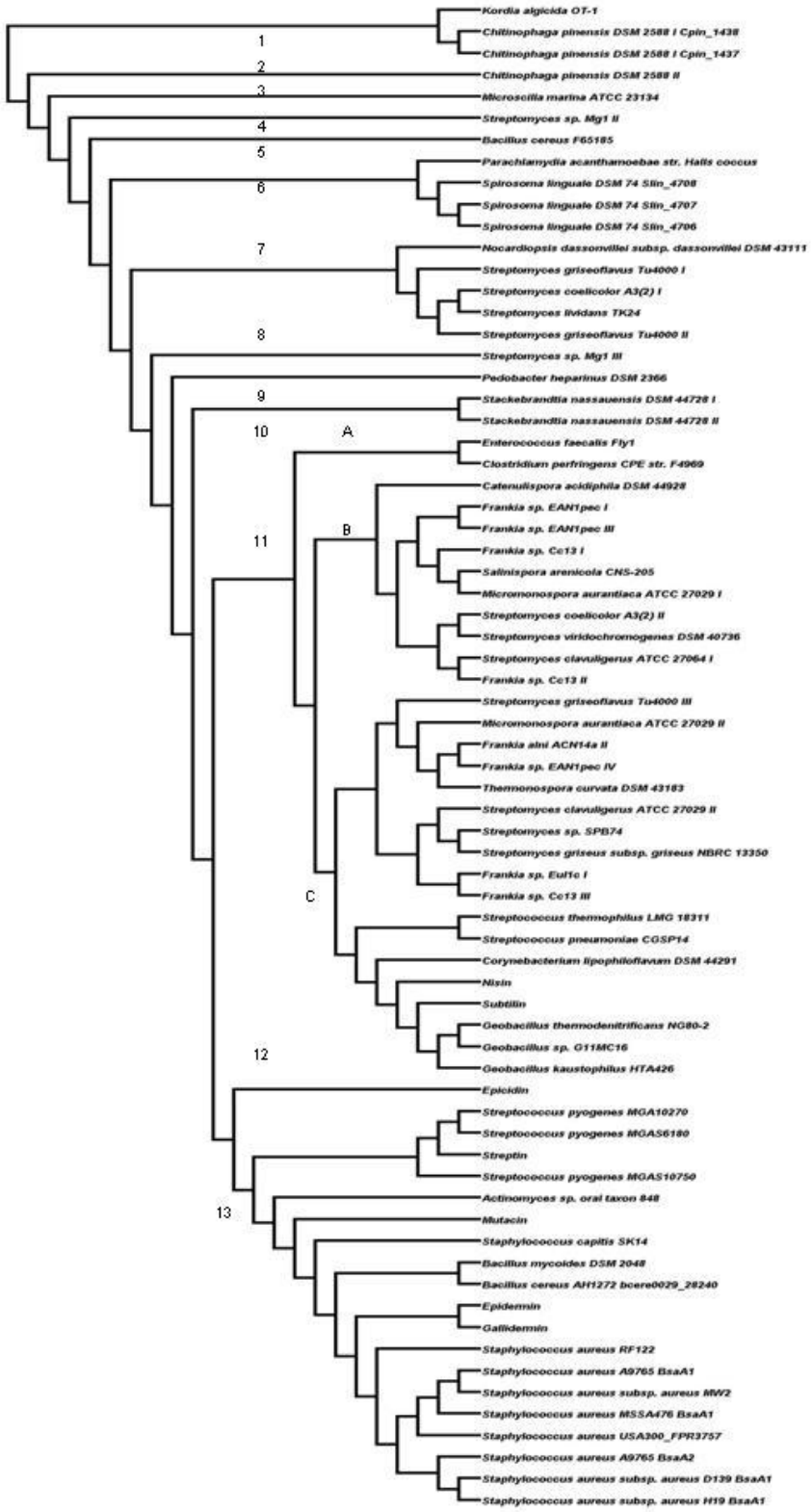

Figure 7

A cladogram of all the LanA prepeptides identified in this study, as well as a number of previously identified LanAs.

3.4.6 Alignment of LanABC

The availability of a significant number of LanA, B and C protein sequences enabled further *in-silico* analysis to identify conserved motifs and residues. Alignment of 66 LanB proteins highlighted a number of conserved motifs which are summarised in **Table 5**. A YxxR motif (corresponding to residues 80-83 of NisB) is conserved in 89% of the LanB enzymes, while a GxG motif (363-365) is present in 92% of LanBs, with the LanB of *C. lipophiloflavum* being exceptional by virtue of lacking both glycine residues. A GRF motif (463-465) is fully conserved in 86% of LanBs with the *Streptomyces* sp. Mg1 III LanB being the only protein to lack this motif. An RxTPFG motif (87-94) is present in 77% of LanBs but is completely absent from the LanBs of *Geobacillus* sp. G11MC16, *Streptomyces* sp. Mg1 III, *M. aurantiaca* and *C. lipophiloflavum*. A FxxxYG motif (342-347) is present in 82% of LanBs and, although present in only 50% of LanBs, a PxxxRxxNV (501-509) motif is at least partially conserved in many such proteins i.e. 94% contain the proline, 71% contain the NV residues and the least conserved is the arginine with 71% conservation. Elsewhere, there is a RFL motif (585-587) conserved in 51% of LanBs, a RYG motif (826-828) conserved in 85% of LanBs and a HxxxNR motif (961-966) in 70% of the dehydratases. In addition to these, there are multiple highly conserved residues such as aspartates at residues 121, 299, 648 and 843, prolines at 612 and 639 and a leucine, tryptophan and phenylalanine at 97, 616 and 840.

Alignment of the LanC protein also revealed several conserved regions (summarised in **Table 6**). Of these, CHG and WCYG motifs were particularly notable. The CHG motif (corresponding to residues 330-333 of NisC) was found

Table 5. Highly conserved residues shared by 66 cluster-associated (including those from the 49 novel clusters referred to in this paper) lanthionine dehydratases (LanB). Residues are numbered according to their position in NisB

From Alignment	NisB Position	% Conservation
Motifs		
YxxR	80-83	89%
RxTPFG	87-94	77%
FxxxYG	342-347	82%
GxG	363-365	92%
GRF	463-465	86%
PxxxRxxNV	501-509	50%
RFL	585-587	51%
RYG	826-828	85%
HxxxNR	961-966	70%
Single Residues		
R	14	86%
D	121; 299; 648; 843	86%; 94%; 94%; 85%
N	145	86%
L	217	97%
P	612; 639	100%; 95%
E	975	89%
W	616	98%
F	840	95%
V	352	83%

Table 6. Highly conserved residues shared by 66 cluster-associated (including those from 49 novel clusters) lanthionine cyclases. Residues are numbered according to their position in NisC

Conserved Residues	NisC Position	% Conservation
Motifs		
LxxG	39-42	83%
YDxxxGxxG	140-148	67%
GxAHGxxG	209-216	83%
WCYG	283-286	95%
CHG	330-332	98%
GxxxGxxGxxLxL	377-389	73%
Single Residues		
G	90	94%
W	258; 401	83%; 92%

to be conserved in 98% of the LanCs. The cysteine^{330} and histidine^{331} residues, which act as ligands to the zinc in the active site of NisC, have been shown to be necessary for enzyme activity (Li and van der Donk, 2007). The WCYG motif (283-286) was present in 95% of the aligned enzymes. Within the WCYG motif, tryptophan^{283} (W) and cysteine^{284} (C) have been shown to be vital to subtilin and nisin biosynthesis (residue numbers refer to location in NisC) (Helfrich et al., 2007, Li and van der Donk, 2007). It has previously been shown that although alanine subsititution of tyrosine^{285} (Y) results in enzyme inactivation, a phenylalanine change is tolerated indicating that the presence of an aromatic ring at this position is of key importance (Li and van der Donk, 2007). In the same study, a preceding arginine residue (Arg^{280}), present in 86% of these enzymes, was found not to be essential for enzyme activity. 92% of LanCs also contained a closely located Gly^{289} residue. The histidine^{212} of another highly conserved motif, GxAHGxxG (209-216; conserved in 83% of LanCs), together with a conserved aspartic acid^{141} (91% of LanCs) are thought to be involved in the electrophilic activation of the carbonyl group of dehydroalanine/dehydrobutyrine or in the protonation of the enolate (thiol substrate) (Li and van der Donk, 2007). The HG of this latter motif was conserved in 98% of the enzymes (the exception being *S. aureus* subsp. *aureus* D139). In addition to these, other motifs of note included LxxG (39-42; conserved in 83% of LanCs), GxxxGxxGxxLxL (377-389; 73%) and YDxxxGxxG (140-148; 67%). Highly conserved single residues include Gly^{90} (94%) and Tryp^{258} and Tryp^{401} (83% and 92% respectively).

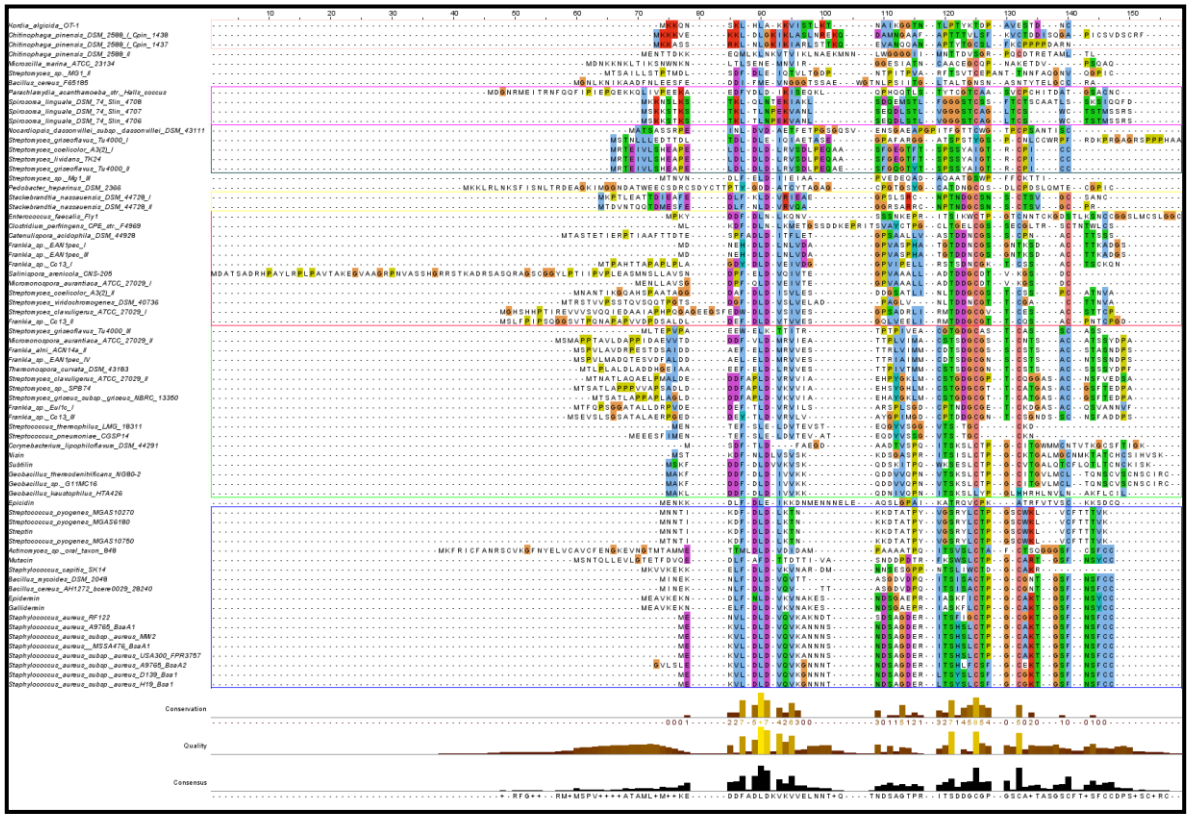

Figure 8. Alignment of the LanA prepeptides identified in this study, as well as a number of previously identified LanAs. This revealed the proposed end of the leader and beginning of the core peptide, between positions 100 and 110. Different groupings based on similar residues are highlighted by coloured boxes.

Although LanA peptides are less conserved than their modification enzymes, some motifs were evident (**Figure 8**). A DLD motif present in the leader region of almost all the phylogroup 13 LanAs is also found in many other LanAs. Indeed, the leucine of this motif is conserved across 93% of the 70 aligned peptides. The only peptides lacking this residue are those from *M. marina*, *B.cereus* F65185, *N. dassonvillei* subsp. *dassonvillei* and *P. heparinus* as well as mutacin 1140. The leader regions from many actinobacteria, and especially those from phylogroup 11, are also distinctive as a consequence of the frequent presence of proline residues.

Within the propeptide, the most highly conserved residues are cysteines corresponding to positions 30 and 34 of the Nisin A prepropeptide (Hsu et al., 2004) which significantly are within the lipid II-binding region of the peptide. These cysteines are each found in 86% of the type 1 LanAs. The *Actinobacteria* in phylogroups 10 and 11 also share a conserved or partially conserved DGCG motif in the propeptide region. A less highly conserved AC motif which is closer to the C terminus is also evident. In addition to motifs which are conserved across prepeptides, a large number of motifs which are conserved within phylogroups are evident.

3.5 Conclusions

The *in-silico* strategy adopted here resulted in the initial identification of 56 proteins which share 20-30% identity with NisC. Further investigation of novel LanC proteins in turn led to the identification of even more homologs, revealing novel lantibiotic/lantipeptide associated clusters and establishing the existence

of subgroups of LanA, B and C proteins. Theoretically, additional homologs could be identified through a continuation of this method but the rate at which new homologs would be identified would begin to level off. The approach taken led to the identification of 49 novel clusters which, prior to this study, had not been the subject of a detailed bioinformatic analysis. While the *in-silico* identification of gene clusters in a strain will not always be confirmed by detection of an associated lantibiotic/lantipeptide, past experience (Begley et al., 2009, Daly et al., 2010, Lawton et al., 2007, McClerren et al., 2006) suggests that there is likely to be a strong correlation. It is thus anticipated that the peptides produced by these gene clusters will represent a valuable resource, as will the associated biosynthetic proteins.

This study reveals new details regarding type 1 lantibiotics and their associated clusters. Type 1 lantibiotics have been predominantly associated with the *Firmicutes*, with the *Actinobacteria*-produced planosporicin and microbisporicin being notable exceptions. It is thus interesting to find type 1 clusters distributed among the genomes of bacteria representing four different phyla, the *Actinobacteria*, *Firmicutes*, *Bacteroidetes* and *Chlamydiae*, which have been isolated from a diverse range of habitats including soil, skin, intestines and the deep-sea. Indeed, based on these investigations, it would appear that such clusters are as common among *Actinobacteria* as they are among *Firmicutes*, with *Streptomyces* and *Frankia* sp. being particularly rich sources. The *Actinobacteria* clusters are, in general, quite similar, typically encoding a LanA, B, C and a methyltransferase. The role of the methyltransferase is not clear but may serve to protect specific serine and

threonine residues from LanB-mediated dehydration. The presence of five clusters within the genomes of five *Bacteroidetes*, a phylum in which bacteriocin production is purportedly quite rare, is particularly noteworthy. However, the sequencing of additional representatives of this species may well reveal this to be a common feature. The *P. acanthamoebae* cluster is unusual by virtue of its presence in a representative of the *Chlamydiae*. Phylogenetic analysis indicates that the LanB and LanC proteins from this strain are closely related to those of several *Firmicutes* and thus the cluster may originally have been acquired from such a source.

The availability of a much larger collection of LanA, B and C sequences for further *in-silico* analysis is also extremely useful for a number of other reasons. In addition to providing greater certainty with respect to the proposed conservation of particular motifs, it also reveals the existence of a greater number of subgroups of sequences than was previously apparent. This is particularly important with respect to LanAs as alignment of these peptides has previously been employed as a means of subgrouping type 1 lantibiotics (Piper et al., 2009, Rea, 2010). Ultimately, the most significant outcome has been the number of new type 1 lantibiotic gene clusters. When one considers that less than 25 type 1 lantibiotics had been identified prior to this study, this represents a major expansion. While the genome sequenced strains themselves can be accessed with a view to purifying the associated peptides and/or utilising the biosynthetic machinery, the information gathered will also encourage researchers to include *Actinobacteria* and *Bacteroiodetes* when carrying out wet lab-based screens for novel lantibiotic producers. A combination of this

approach and analysis of newly generated bacterial genome sequence data will ensure that many more lantibiotics and lantipetides will soon be discovered which are associated with unusual microorganisms and a wide variety of environments.

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CHAPTER IV

Sequencing-based analysis of the bacterial and fungal composition of kefir grains and milks from multiple sources

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4.1 ABSTRACT

Kefir is a fermented milk-based beverage to which a number of health-promoting properties have been attributed. The microbes responsible for the fermentation of milk to produce kefir consist of a complex association of bacteria and yeasts, bound within a polysaccharide matrix, known as the kefir grain. The consistency of this microbial population, and that present in the resultant beverage, has been the subject of a number of previous, almost exclusively culture-based, studies which have indicated differences depending on geographical location and culture conditions. However, culture-based identification studies are limited by virtue of only detecting species with the ability to grow on the specific medium used and thus culture-independent, molecular-based techniques offer the potential for a more comprehensive analysis of such communities. Here we describe a detailed investigation of the microbial population, both bacterial and fungal, of kefir, using high-throughput sequencing to analyse 25 kefir milks and associated grains sourced from 8 geographically distinct regions. This is the first occasion that this technology has been employed to investigate the fungal component of these populations or to reveal the microbial composition of such an extensive number of kefir grains or milks. As a result several genera and species not previously identified in kefir were revealed. Our analysis shows that the bacterial populations in kefir are dominated by 2 phyla, the Firmicutes and the Proteobacteria. It was also established that the fungal populations of kefir were dominated by the genera *Kazachstania*, *Kluyveromyces* and *Naumovozyma*, but that a variable sub-dominant population also exists.

4.2 INTRODUCTION

Kefir is a fermented milk-based beverage. It is a viscous, self-carbonated, acidic drink, which contains a low alcohol percentage and is believed to have originated in the Caucasian mountains some 2000 years ago. The milk is fermented by a solid, cauliflower-like, polysaccharide matrix known as a kefir grain, which is reused to start subsequent fermentations. The grain is primarily composed of bacterially-produced kefiran (La Riviere and Kooiman, 1967), which contains within it a complex consortium of bacteria and yeast that work in symbiosis to ferment the milk (Farnworth, 2005).

The microbial composition of kefir and kefir grains is believed to vary depending on geographic, climatic and cultural conditions as well as the diversity of local species of wild yeasts and bacteria. Culture-based analyses suggest that bacteria constitute the majority, up to 90%, of the population in the grain (Simova et al., 2002). Such culture-based studies have also revealed that the bacterial composition of kefir predominantly consists of the lactic acid bacteria (LAB) *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus* as well as acetic acid bacteria from the genus *Acetobacter* (Witthuhn et al., 2005, Chen et al., 2008, Kesmen and Kacmaz, 2011). Bacteria contribute to the production of lactic acid, which preserves the milk, and produces various antimicrobial and flavour compounds (e.g. acetaldehyde) in addition to other metabolites (e.g. extracellular polysaccharides), free amino acids and vitamins (Guzel-Seydim et al., 2011). Other studies have revealed that the yeast component of kefir consists of *Kluyveromyces*, *Saccharomyces*, *Candida* and *Torulaspora* (Wyder et al., 1997, Simova et al., 2002, Lin et al., 1999, Loretan et al., 2003, Angulo et al.,

1993). Other yeast which have less frequently been associated with kefir include *Pichia*/*Issatachenkia* (Lin et al., 1999, Latorre-Garcia et al., 2007), *Brettanomyces*/*Dekkera* (Wyder et al., 1997, Pintado et al., 1996), *Zygosaccharomyces* (Witthuhn et al., 2005) and *Yarrowia* (Loretan et al., 2003), while recent molecular-based studies have detected the presence of *Kazachstania* (Zhou et al., 2009, Magalhaes et al., 2011, Gao et al., 2012). Yeasts perform the vital role of alcohol and carbon dioxide production in the milk, and produce metabolites thought to be important with respect to mouthfeel and taste (Kwak et al., 1996). Ultimately, following a 24 hour fermentation, culture-based approaches indicate that lactococci/streptococci are present at 10^8-10^9 ml^{-1}, *Leuconostoc* at 10^7-10^8 ml^{-1}, acetic acid bacteria at 10^5-10^6 ml^{-1}, lactobacilli at 10^5-10^6 ml^{-1} and yeasts at 10^6-10^7 ml^{-1} (Rea et al., 1996, Garrote et al., 2001).

Despite the undoubted value of the aforementioned studies, culture-based analyses are limited by virtue of only detecting species with the ability to grow on the specific medium used. Thus, culture-independent techniques have the potential to provide a more accurate and in-depth analysis. Although culture-independent techniques such as Sanger sequencing (Gao et al., 2012, Tas et al., 2012, Latorre-Garcia et al., 2007, Wang et al., 2008) and DGGE (Garbers et al., 2004, Magalhaes et al., 2011, Zhou et al., 2009) have been employed to explore the kefir population, the application of high-throughput DNA sequencing to investigate such microbial ecosystems has been a particularly significant development. This strategy has been employed to study the microbial composition of a number of fermented food environments such as cheese (Quigley et al., 2012b, Masoud et al., 2011), fermented fish (Koyanagi et

al., 2011, Roh et al., 2010), fermented vegetables (Park et al., 2012), rice bran (Sakamoto et al., 2011) and pearl millet slurry (Humblot and Guyot, 2009). Indeed, high-throughput DNA sequencing was also recently utilised to gain a more comprehensive understanding of the bacterial population of one Irish kefir grain and milk, and three Brazilian kefir grains (Dobson et al., 2011, Leite et al., 2012).

The benefits of gaining a better appreciation of the microbial composition of kefir and kefir grains relate to the fact that the history of kefir has long been linked to its purported health benefits. Preliminary studies have shown kefir to reduce lactose intolerance symptoms, stimulate the immune system, lower cholesterol, and to have antimutagenic and anticarcinogenic properties (Guzel-Seydim et al., 2011). It is thus unsurprising that, as a functional dairy food, kefir has become the focus of increased study in recent years. While some of the health benefits thought to be derived from the consumption of kefir may be associated with the biochemical changes that occur within the milk, such as the production of organic acids, bioactive peptides etc., the microbial species present may also have health-promoting attributes. Notably, genera to which many strains with health-beneficial or probiotic properties are assigned, such as *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus* and *Streptococcus*, have been isolated from kefir in the past (Parvez et al., 2006, Tas et al., 2012). From a fungal perspective, strains of the yeast *Saccharomyces boulardii* have been established to possess health-promoting properties in clinical trials (Czerucka et al., 2007, Desreumaux et al., 2011, Foligne et al., 2010). Strains of *Saccharomyces cerevisiae*, as well as

Kluyveromyces lactis/*Candida kefyr*, commonly associated with kefir, also show potential in this regard (Etienne-Mesmin et al., 2011, You et al., 2006, Kumura et al., 2004). Conversely, however, *Candida kefyr* has been shown to cause oesphagitis in a patient with squamous cell carcinoma (Listemann et al., 1998).

Aside from identifying potentially health-promoting populations, the commercialisation of kefir production could benefit from gaining a detailed understanding of the associated microbial populations. There is also a need to assess the heterogeneity of these populations across a large number of grains and, in particular, to employ molecular approaches to better characterise the associated yeast populations. In light of these requirements, the aim of this study was to use high-throughput sequencing techniques to provide in-depth analysis of the microbial consortium of 25 distinct kefir grains and milks obtained from a variety of different sources in order to minimise any geographic bias that might influence the floras. This study represents the first occasion upon which this technology has been applied to such an extensive number of kefir samples and is the first study of its kind to reveal the fungal component of kefir.

4.3 MATERIALS AND METHODS

4.3.1 Culture maintenance

9 Irish kefir grains were recultured from -80°C storage within the Teagasc Culture Collection by fermenting in 10% reconstituted skimmed milk (RSM), which had been sterilized at 115°C for 15mins. These were originally acquired from housewives across the country (Rea et al., 1996), and for the purposes of

this study were designated IR1, 2, 3, 4, 5, 6, 8, 9 and 10. An additional 16 grains were obtained from individual and commercial suppliers from a number of different locations (**Table S1**), and cultivated under uniform conditions. Samples from the United Kingdom were designated UK1 to UK5 and samples from the United States were designated US1, 2, 3 and 5. Other kefir grains were sourced from Spain (Sp1), Belgium (Bel1), France (Fr1), Italy (It1), Canada (Ca1) and Germany (Ger1 and Ger2). Cultures were maintained at room temperature and inoculated into fresh milk 3 times per week, for a minimum of 4 months prior to extraction.

4.3.2 Metagenomic DNA extraction

100mls of 10% RSM was inoculated with 1g of kefir grain and fermented at 25°C for 24 hours, the time at which kefir is most frequently prepared. To extract DNA from the kefir, 1.8mls of fermented milk was centrifuged to generate a pellet which was suspended in 450ul of lysis buffer P1 from the Powerfood Microbial DNA Isolation kit (MoBio Laboratories Inc, USA). The resuspended pellet was subjected to enzymatic digestion with enzymes mutanolysin (100U/ml) and lysozyme (50µg/ml) at 37°C for 1 hour, followed by proteinase K (250µg/ml) digestion at 55°C for 1 hour. Extraction was optimised with a 10 minute 70°C incubation (Quigley et al., 2012a) prior to mechanical lysis using the Qiagen TissueLyser II (Retsch®). The Powerfood Microbial DNA Isolation kit was then used as per the manufacturer's instructions. Pure DNA was eluted in HPLC grade sterile water. DNA from kefir grain was isolated using a modified phenol-chloroform-based extraction procedure (Garbers et al., 2004).

4.3.3 DNA amplification and pyrosequencing

Metagenomic DNA extracts were used as a template for PCR amplification, with BioMix red (Bioline) which has a reported error rate of $2x10^{5}$ errors/bp (Lundberg et al., 1991). PCR amplification of the V4-V5 variable region (408 bp) of the 16S rRNA gene was performed using the universal primers F1 (5'-AYTGGGYDTAAAGNG) and R5 reverse (5'-CCGTCAATTYYTTTRAGTTT) to facilitate an investigation of the bacterial component of the microbial populations (Claesson et al., 2010). Unique multiplex identifier adaptors, 8 bp in length, were attached between the 454 adaptor sequences and the forward primers to facilitate the pooling and subsequent differentiation of samples (Cole et al., 2009). Tagged universal primers were also used to amplify fungal DNA from the variable ITS-1 rRNA region (Buee et al., 2009). In this instance the forward primer ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA) and ITS2 reverse (5'-GCTGCGTTCTTCATCGATGC) generated PCR products of *circa* 410 bp. The PCR conditions used for 16S amplification were 94°C denaturation for 2 min, 35 cycles of 94°C for 1 min (denaturation), 52°C for 1 min (annealing) and 72°C for 1 min (extension) followed by a final 72°C for 2 mins. The PCR conditions used for ITS amplification were 94°C denaturation for 4 min, 35 cycles of 94°C for 30 seconds (denaturation), 50°C for 1 min (annealing), and 72°C for 1 min and 30 seconds (extension). A final annealing step of 72°C for 10 mins was performed. All DNA was subject to a 10 min hotstart at 94°C prior to PCR amplification. Amplicons generated from three PCR reactions/template DNA were pooled and cleaned using the Agencourt AMPure® purification system (Beckman Coulter

Genomics, Takeley, UK). Purified products were quantified using the Nanodrop 3300 Fluorospectrometer (Thermo Scientific) and the Quant-iT™ Picogreen® dsDNA Assay kit (Invitrogen). Equal concentrations of 16S or ITS amplicons were pooled, AMPure cleaned and assessed by an Agilent 2100 Bioanalyser (Agilent Technologies) to determine purity and to ensure the absence of primer dimers. Sequencing of the 16S rRNA V4-V5 and ITS1 rDNA amplicons was performed using a 454 Genome Sequencer FLX Titanium System (Roche Diagnostics Ltd) at Teagasc Food Research Centre, Moorepark, according to 454 protocols.

4.3.4 Pyrosequencing data analysis

Raw sequences were quality trimmed and filtered using the Qiime Suite of tools (Caporaso et al., 2010); any reads not meeting the quality criteria of a minimum quality score of 25 and sequence length shorter than 150bps for 16S amplicon reads and 200bps for ITS amplicon reads were discarded. The maximum homopolymer limit was increased to 10 for ITS amplicons as ITS sequences are known to harbour long homopolymer runs. Trimmed fasta sequences were assessed by BLAST analysis against the SILVA database (version 100) for 16S reads (Pruesse et al., 2007). The ITS-1 specific database, ITSoneDB, was used to BLAST all ITS sequences (Santamaria et al., 2012). BLAST outputs were parsed using MEGAN (Huson et al., 2007) with a bit-score of 86 was employed for 16S ribosomal sequence data, and a bit-score of 35 was used for ITS sequence data. The QIIME suite of programs was used to calculate alpha diversity including Chao1 richness, Shannon diversity, Simpson index, Phylogenetic Diversity and Observed species (Caporaso et al., 2010). Sequencing depth was estimated

using rarefaction analysis. QIIME was also used to generate weighted UniFrac, unweighted UniFrac and Bray-Curtis distances matrices. Principal Co-ordinate Analysis plots based on these distance matrices were generated with Qiime and visualised using King (Chen et al., 2009b). Statistically significant differences between the combined kefir grains and combined fermented milks were determined by the non-parametric Mann-Whitney test using the Minitab® statistical package. Reads were deposited in the SRA database under the accession number ERP002650.

4.4 RESULTS

4.4.1 The bacterial population of kefir milk is more consistent and less diverse than that of the corresponding grains

Post-quality filtering, 106,235 and 136,815 reads for 23 grain and the corresponding 23 milk samples, respectively remained, equating to an average of 4,619 reads for each grain sample and 5,949 reads per milk sample.

Chao1 values (reflective of OTU/species richness), Shannon and Simpson indices (to determine species diversity) as well as the Phylogenetic Diversity and Observed Species numbers were all calculated (**Table S2**). Rarefaction curves, calculated at 97% similarity, are approaching parallel to the x-axis for all samples, indicating sufficient reads were obtained to adequately assess the population (**Figure S1**). Box-plot analysis suggests that the bacterial population in kefir milk is generally less diverse than that present in the kefir grains (**Figure S2**), where the median value (black bar) for milk was lower in all metrics, with

the exception of the Shannon index. The only significant difference between the grain and milks was in Phylogenetic Diversity (p<0.001).

Principal Co-ordinate Analysis plots were generated based on the unweighted UniFrac distance matrix (**Figure 1AB**), the only tree-based metric. From this analysis, it was evident that there was no clustering amongst kefir populations from different countries (**Figure 1AB**), and correlated with the other distance matrices (data not shown). Procrustes analysis indicated that the ordinations of kefir and kefir grains were not related to each other (M^2 = 0.924, p = 0.644, **Figure 2A**). The similarities between kefir grain communities were not the same as the similarities between kefir communities.

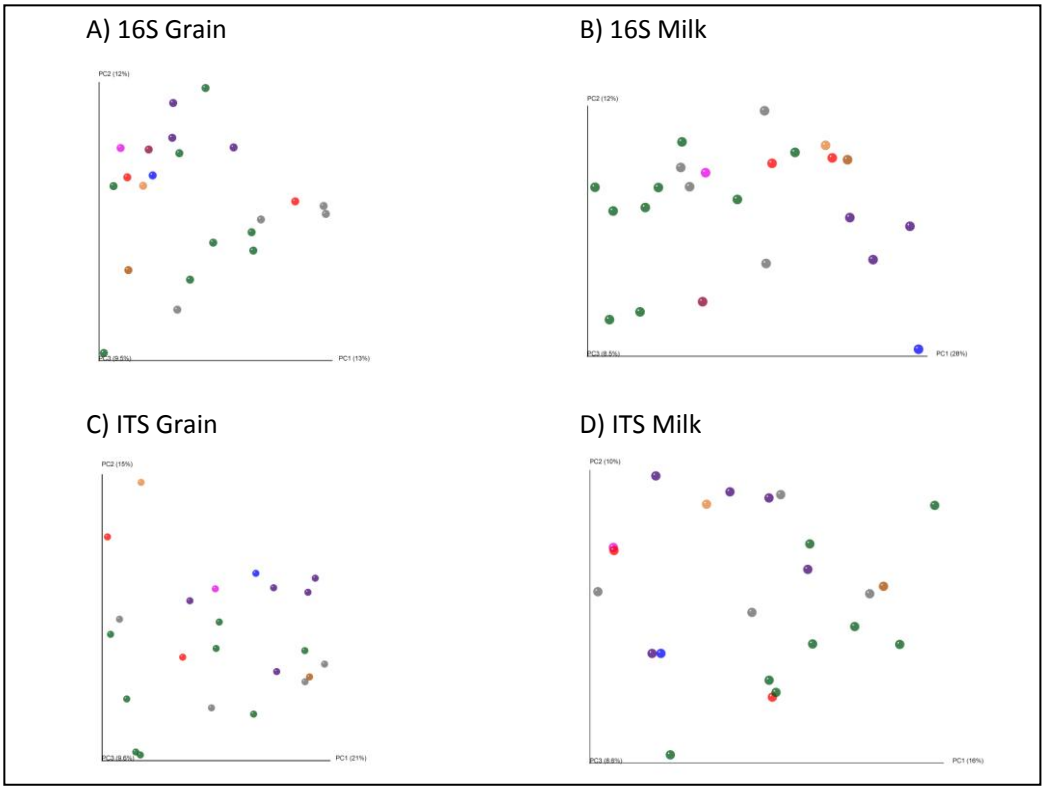

Figure 1: Principle Coordinate Analysis (PCoA) plots, based on unweighted UniFrac distance matrices, show the diversity within bacterial populations from kefir grains (A) and kefir fermented milk (B) and fungal grain (C) and milk (D) populations. Green = Irish kefir, Orange = Belgian kefir, Light Brown = Spanish kefir, Red = German kefir, Grey = US kefir, Pink = Italian kefir and Purple = UK kefir.

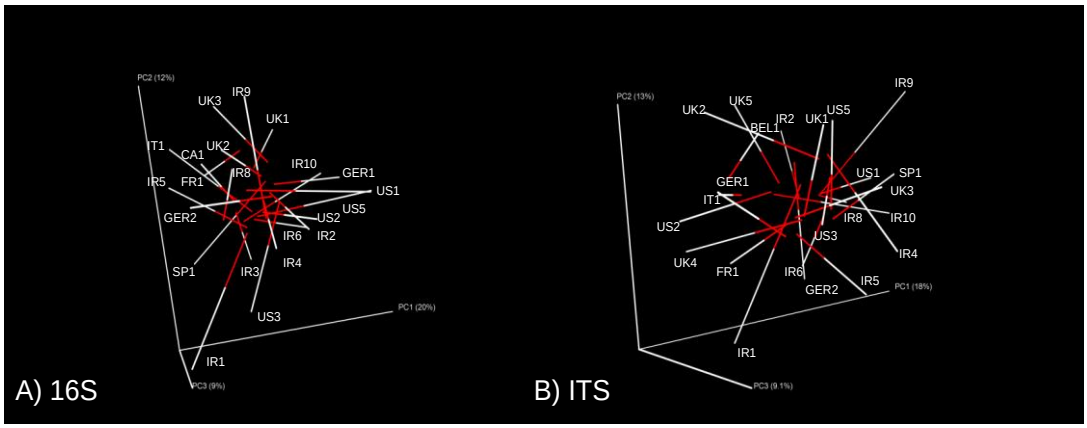

Figure 2: Procrustes imaging of unweighted UniFrac distance matrices highlight the diversity amongst the 16S bacterial component (A) and fungal component (B) of the different kefir samples. The two different sample types are linked with a bar (white represents grain flora; red represents milk flora). The direction of each axis is arbitrary.

4.4.2 The alpha diversity of fungal populations in kefir milks and grains vary but the beta diversity of kefir grains is greater than that of milks

Post quality filtering a combined total of 118,879 and 118,976 reads corresponding to 23 grain and the corresponding 23 milk populations, respectively, were generated. This equated to an average read number of 5,167 and 5,173 per grain and milk sample, respectively.

Alpha diversity values established that there is a naturally low diversity in the kefir grains and milks (**Table S3**). Box-plot analysis of Chao1, Observed Species and Phylogenetic Diversity indices suggest diversity is greater in the kefir milk than in the grains (**Figure S3**). However, statistical difference between the two was limited to Phylogenetic Diversity ($p<0.001$). Rarefaction curves are approaching parallel to the x-axis for all samples, suggesting a sufficient depth of sequencing (**Figure S4**).

To measure beta diversity, Principal Co-Ordinate Analysis Plots were generated based on unweighted UniFrac distance matrices (**Figure 1CD**), but no clustering was evident. Procrustes analysis of the two PCoAs again shows that the similarities between the kefir grains and kefir milks were not the same, with respect to the fungal populations ($M^2 = 0.855$, $p = 0.139$, **Figure 2B**).

4.4.3 The kefir grains and associated kefir-fermented milks are dominated by a relatively small number of bacterial genera

Four bacterial phyla were detected in the kefir grain. These were the Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. Of these, the Bacteroidetes were not identified among the milk bacteria, and were found in

only 9 grains. Across both the grains and milks, the two dominant phyla were the Firmicutes and the Proteobacteria. Indeed most grain samples contained a majority (>50%) of Firmicutes, with the exception of Ir6, which possessed 69.14% Proteobacteria. Proteobacteria were not detected in grains Ca1, Ir9 or UK3. Among the milk samples, Ir1, Ir5, Ir10, US1 and Ir8 were also unusual by virtue of containing a bacterial population dominated by Proteobacteria, which in the case of Ir8, was as high as 90.4%. Milks corresponding to Fr1 and UK3 lacked Proteobacteria. No consistent shift (increase or decrease) in Proteobacteria populations from kefir grain to kefir milk was evident (**Table S4**; **Table S5**). Bacteria corresponding to the phylum Actinobacteria were detected in only two grains, Ir9 (5.87%) and UK2 (0.24%). The relatively high percentage of Actinobacteria in Ir9 may explain why the corresponding kefir milk was the only sample in which Actinobacteria were detected (0.26%). There was a significantly greater abundance of unassigned phyla among the total grains than the total milks (p<0.001).

At the family level, the greater bacterial diversity (in terms of number of different families) within the grain is evident. Only five families of bacteria were detected in the milk whereas twelve were identified in the grain samples (**Tables S4-S5**). The grains were predominantly composed of *Lactobacillaceae*, which accounted for >50% of the populations in all but grain Ir6. The other major family were the Proteobacteria-associated *Acetobacteraceae*. Other families detected were *Streptococcaceae* (19 grains), *Leuconostocaceae* (4 grains), *Lachnospiraceae* (16 grains), *Ruminococcaceae* (8 grains), *Bifidobacteriaceae* (2 grains), *Clostridiaceae* (2 grains), *Propionibacteriaceae* (2

grains), *Bacteroidaceae* (2 grains), *Enterococcaceae* (1 grain) and *Rikenellaceae* (1 grain) (**Table S4**). Among the other families, *Streptococcaceae* were detected in 19 of the 23 grains with the highest proportions found in UK2 (5.12%). *Leuconostocaceae* were found in only four of the grain samples (Bel1, 0.31%; Fr1, 0.13%; UK1, 0.29%; UK2, 0.51%). *Lachnospiraceae* were found in 16 grains from highest abundance in Ir9 at 0.51%, to lowest in US2 at 0.09%. *Ruminococcaceae* were found in 8 samples, from a high of 8.21% in Bel1 to a low of 0.08% in UK2. *Bifidobacteriaceae* were present in only 2 grains (0.81% in Ir9, and 0.10% in UK2), as were *Clostridiaceae* (Ger1, 0.39% and US2, 0.12%), *Propionibacteriaceae* (Ir9, 4.94% and UK2, 0.13%) and *Bacteroidaceae* (UK2 and UK3, 0.08%). *Enterococcaceae* (Ir9, 0.22%) and *Rikenellaceae* (US2, 0.07%) were present in only one grain each. The bacterial populations within the milks were dominated by *Streptococcaceae*, which were found at greater proportions in the kefir milks than in the grains (p<0.001), and form the dominant population (>50%) in 13 samples. Ir3, Ir8 and US1 were notable exceptions by virtue of containing 10.16%, 2.87% and 10.91% *Streptococcaceae*, respectively. In its place, Ir3 has the highest proportions of *Lactobacillaceae* at 60.51%, whereas Ir8 and US1 had the two highest proportions of *Acetobacteraceae* with 90.41% and 77.06%, respectively. However, in general, proportions of *Lactobacillaceae* were significantly lower in the milks than in the corresponding grains (p<0.001). The overall average proportion of *Acetobacteraceae* did not change significantly from the grains to the corresponding milks despite the fact that large increases were evident in some cases (i.e. the aforementioned Ir8 and US1 as well as Ir1 and Ir5). Proportions of *Leuconostocaceae* were detected in all kefir milk

samples (in contrast to just 4 grain samples), reflecting a significant overall increase (p<0.001). *Propionibacterineae* was found in a single milk sample, Ir9, at 0.22%, which is a reduction from the 4.94% in corresponding grain. The proportions of unassigned reads were <1% in almost all grain and milks, with the exception of 1.02% in the grain of Ca1 (**Table S4**; **Table S5**).

The distribution pattern at the genus levels closely resembles that observed at family level, with one genus frequently corresponding to all reads assigned to that family (**Figure 3**). *Lactobacillus* (p<0.001) is the dominant genus in the grain with proportions of *Lactococcus* and *Leuconostoc* being significantly higher in the kefir milks (p<0.001). Once again, the differences in proportions and distribution of *Acetobacter* (of family *Acetobacteraceae*) in the grain and milk were numerically, but not statistically, different.

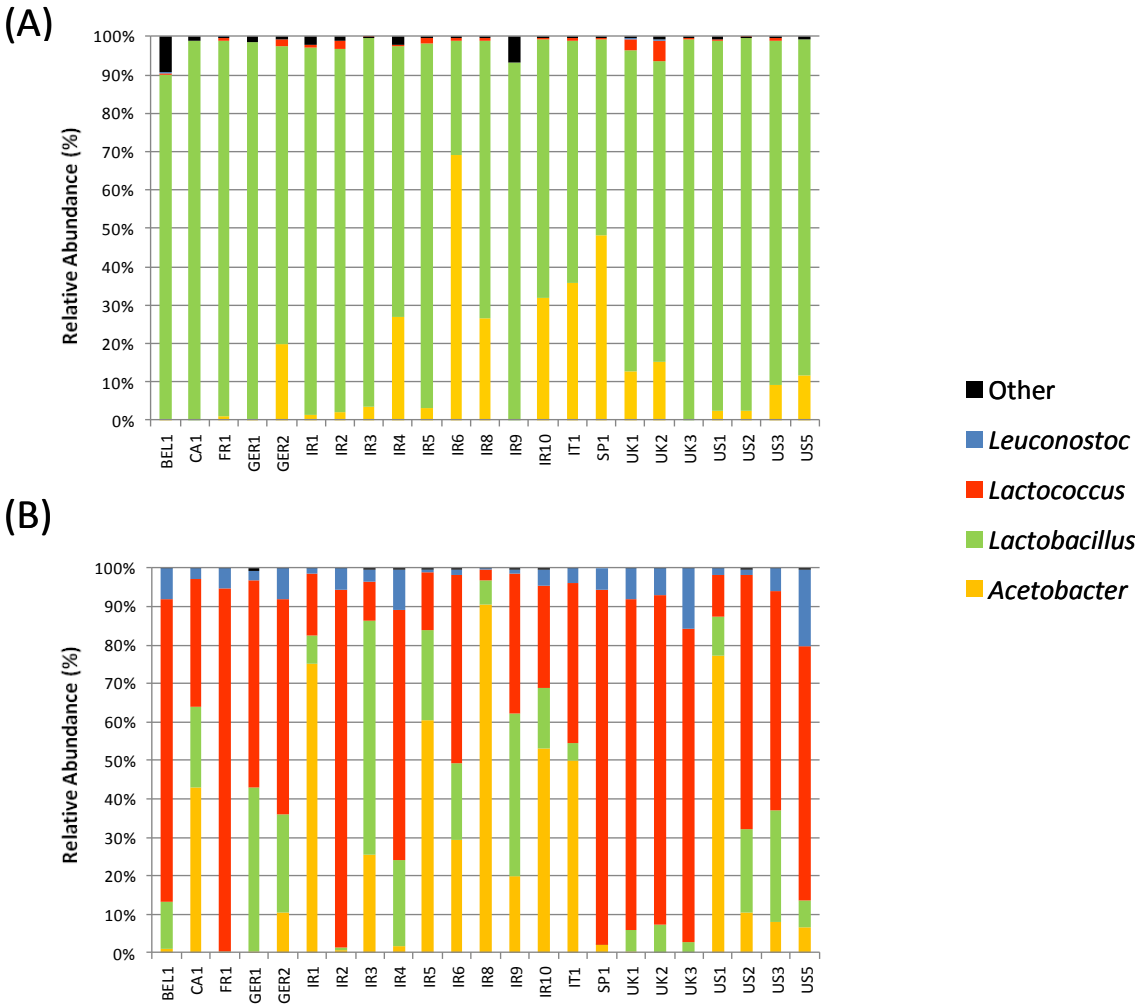

Figure 3: 16S phylogenetic composition of the bacterial component of the kefir grain (A) and kefir fermented milk (B) at genus level.

4.4.4 ITS sequencing provides a detailed insight into the fungal composition of kefir grains and associated kefir-fermented milks

The only fungal phylum assigned in the grain was Ascomycota, the largest phylum of the fungal kingdom. Ascomycota were also shown to dominate within the kefir milk, ranging from a high of 100% in Ger1 to a low of 89.38% in Ir10 (**Table S6**; **Table S7**). Basidiomycota, the other phylum belonging to the subkingdom Dikarya, was found in 9 milk samples at relatively low read numbers. 9 of the milk samples also harboured trace amounts of uncultured fungi. The lower diversity in the grain is again evident at the family level where all but one sample (Sp1) contain >99% *Saccharomycetaceae*. The overall average proportion of *Saccharomycetaceae* is significantly lower in the milks (p<0.001), but still correspond to >99% of reads in 16 of the 23 samples. The fungal composition of kefir milk Sp1 was unusual by virtue of containing 34.27% *Pichiaceae*. In contrast, the next highest proportion of *Pichiaceae* was 0.48% (in milk UK3). Other fungal families detected in both the kefir milks and grains were *Davidiellaceae* and *Trichocomaceae*. *Herpotrichiellaceae*, *Teratosphaeriaceae*, *Valsaceae*, *Debaryomycetaceae*, *Phaffomycetaceae*, *Malasseziaceae*, *Bondarzewiaceae*, *Dermataceae*, *Pezizaceae*, *Ganodermataceae*, *Tricholomataceae*, *Tremellomycetes*. In addition, *Wallemiomycetes* were only detected in the milks whereas *Dothioraceae* were only detected in the grains.

The most common fungal genus across both the kefir milk and grains was *Kazachstania* (**Figure 4**). This genus was detected in all samples except kefir grain Ger1. Given that the corresponding milk contained *Kazachstania* at a proportion of 5.68%, it would seem likely that this grain did contain

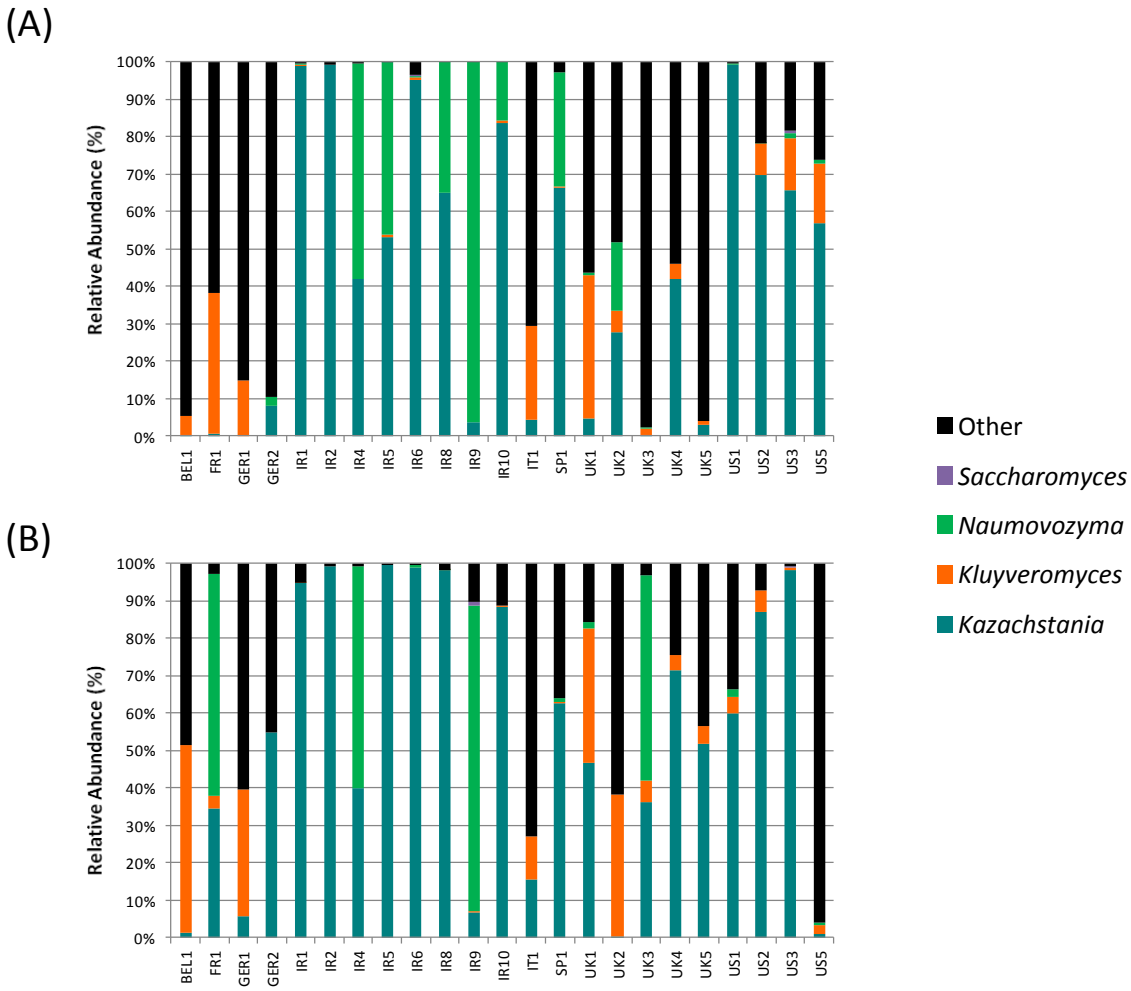

Figure 4: ITS phylogenetic composition of the fungal component of the kefir grain (A) and kefir fermented milk (B) at genus level.

Kazachstania at levels below the limit of detection for this study. The proportions of *Kazachstania* were >50% in 11 of the grains and in 13 of the milks and was highest in grains Ir2 and US1 (99.40% and 99.25%, respectively) and the milks Ir2 and US3 (99.20% and 98.07%, respectively). In contrast, proportions were low in grains Bel1 and UK3 (0.24% and 0.39%, respectively) and milks UK2 and US5 (0.44% and 0.89%, respectively). *Naumovozyma* was the second most prevalent fungal genus, being present in 16 grains and 10 milk samples, accounting for 13.09% total grain reads, and 9.98% total milks reads. Proportions of *Naumovozyma* varied from being dominant in Ir9 (96.02%, grain; 81.87%, milk) and Ir4 (57.56%, grain; 59.41%, milk) to sub-dominant in Ger2 (2.46%, grain; 0%, milk) and US1 (0.18%, grain; 1.81%, milk), amongst others. Notably, although no *Naumovozyma* were detected in grain Fr1, this genus became dominant in the resultant kefir milk (59.3%), again suggesting the presence of *Naumovozyma* in the grain below the detection threshold. The third most commonly assigned genus was *Kluyveromyces*, which was detected in 17 of the grains and 18 of the milks, accounting for 7.6% and 7.32% of total grain and milk reads respectively. Although *Kluyveromyces* was present at a high of 50.16% in the milk of Bel1, this genus was more frequently present at sub-dominant proportions, with a detected low of 0.05% in the milk of Ir1. At genus level, many of the reads corresponding to the *Saccharomycetaceae* could not be reliably assigned. These corresponded to >50% of reads corresponding to grains Bel1, Fr1, Ger1, Ger2, It1, UK1, UK3, UK4 and UK5 and milks Ger1, It1, UK2 and US5. This is likely a result of such high similarity amongst ITS sequences that they cannot be reliably separated and assigned. Despite numerical differences

in the proportions of the different fungal genera present in the kefir grains and milks, the only significant difference related to a higher proportion of *Dekkera* in the milks than in the grains (p=0.004). The kefir milks also contained a larger number of different genera, often at trace levels, which were not detected in the corresponding grains. These included *Zygosaccharomyces*, *Wallemia*, *Eurotium*, *Microdochium*, *Cryptococcus*, *Teratosphaeria*, *Debaromyces*, *Cyberlindnera*, *Malassezia*, *Heterobasidion*, *Neofabraea*, *Peziza*, *Ganoderma*, *Mycena* and *Dioszegia*. *Penicillium* and *Aureobasidium* were each detected in only a single instance, i.e. in kefir grain Sp1 (0.13%) and grain UK3 (0.09%), respectively.

Unlike the 16S reads which are subject to a high level of sequence homology, the ITS reads were sufficiently dissimilar to enable assignment to species level. **Table 1** shows the total number of different species identified and whether there has been a previous association with kefir. The population profile at species level strongly mirrors that at genus level. The most common species, *Kazachstania unispora*, was present in 20 grains and all milks. All reads from the *Kluyveromyces* and *Naumovozyma* genera were assigned to the species *Kluyveromyces marxianus* and *Naumovozyma castelli*, respectively (**Table S6**; **Table S7**). Although the *Saccharomyces* genus was identified in small amounts in a number of grains and milks, only those in Ir5 were assigned at the species level (to *Saccharomyces cerevisiae*).

Species (Teleomorph)	Anamorph	Synonym	Previous Kefir Association
Kazachstania barnettii	N/A	*Saccharomyces barnettii*	No
Kluyveromyces marxianus	*Candida kefyr*	*Kluyveromyces fragilis, Candida pseudotropicalis*	Yes (Zhou et al., 2009)
Kazachstania unispora	N/A	*Saccharomyces unisporus*	Yes (Zhou et al., 2009, Leite et al., 2012)
Naumovozyma castelli	N/A	*Saccharomyces castellii, Naumovia castellii*	No
Saccharomyces cerevisiae	*Candida robusta*	*Saccharomyces oviformis, Saccharomyces italicus*	Yes (Zhou et al., 2009, Angulo et al., 1993)
Davidiella tassiana	*Cladosporium herbarum*	*Mycosphaerella tulasnei, Mycosphaerella tassiana*	No
Penicillium sp. *Vega* 347	N/A	N/A	No
Pichia kudriavzevii	*Candida acidothermophilum*	*Issatchenkia orientalis, Candida krusei,*	Yes (Gao et al., 2012)
Pichia fermentans	*Candida lambica*	*Candida fimetaria, Mycoderma lambica, Pichia sp.* AWRI 1271	Yes (Lin et al., 1999) (Chen et al., 2005)
Dekkera anomala	*Brettanomyces anomalus*	N/A	Yes (Wyder et al., 1997)
Dekkera bruxellensis	*Brettanomyces bruxellensis*	*Brettanomyces custersii*	No
Zygosaccharomyces lentus	N/A	N/A	No
Eurotium amstelodami	*Aspergillus amstelodami*	*Aspergillus vitis*	No
Wallemia sebi	N/A	N/A	No
Microdochium nivale	N/A	*Fusarium nivale*	No
Cryptococcus sp. *Vega* 039	N/A	N/A	No
Teratosphaeria knoxdaviesii	N/A	N/A	No
Cyberlindnera jadinii	*Candida utilis*	*Pichia jadinii, Hansenula jadinii, Torula utilis, Torulopsis utilis*	No
Malassezia pachydermatis	N/A	N/A	No
Heterobasidion annosum	N/A	N/A	No
Peziza campestris	N/A	*Kimbropezia campestris*	No
Ganoderma lucidum	N/A	N/A	No
Dioszegia hungarica	N/A	*Bullera armeniaca, Cryptococcus hungaricus*	No

Table 1: List of fungal species identified in the study, listed in teleomorph form with anamorph or synonym names and previous kefir association

4.5 DISCUSSION

The study represents the most comprehensive investigation of the microbial population of kefir (both grains and milk) to date. This analysis was facilitated by high-throughput sequencing of 16S rRNA (bacteria) and, for the first time, ITS (fungi) amplicons, generated from a considerably larger collection of samples than has been employed heretofore. The number of reads compare well with previous studies i.e. Dobson *et al*. generated a combined total of 17,416 V4 16S rRNA (4,883 reads for the interior grain, 3,455 reads for the exterior grain and 9,078 reads for the milk fermentate; (Dobson et al., 2011)) while Leite *et al*. generated a total of 14,314 16S rDNA reads (2,641, 2,690 and 8,983 reads for the three grains sequenced, respectively (Leite et al., 2012)). In each index, alpha diversity values were reflective of a naturally low diversity and a homogeneity between kefir samples, relative to other environmental analyses, and rarefaction patterns were consistent with that of previous kefir studies (Dobson et al., 2011, Leite et al., 2012).

16S rRNA profiling revealed that the bacterial population of kefir milks tested is composed of Actinobacteria, Firmicutes and Proteobacteria, with Bacteroidetes also being detected in the grain. The kefir grains were dominated by *Lactobacillaceae*/*Lactobacillus*, establishing that this pattern, which was previously noted in high-throughput sequencing-based studies of a much smaller number of kefirs (Simova et al., 2002, Dobson et al., 2011), is consistent. In contrast, *Streptococcaceae* dominate in the kefir milk. More specifically, lactococci dominate as other genera from this family were not detected. This contrasts with a subset of previous studies in which *Streptococcus* species have

been identified (Simova et al., 2002, Yuksekdag et al., 2004, Leite et al., 2012). The next most common LAB were *Leuconostoc* spp.; *Leuconostoc* have been associated with kefir on a number of previous occasions (Lin et al., 1999, Garrote et al., 2001, Mainville et al., 2006, Motaghi et al., 1997), but the data presented here reveals for the first time that the proportions of this genus increase considerably in the milk relative to the grain where they may significantly impact the sensory profile of kefirs. *Acetobacteraceae* (genus *Acetobacter*) were also identified as major components of the bacterial population of many grains despite having been identified in some (Garrote et al., 2001, Miguel et al., 2010), but not all, previous kefir studies. However, given that kefir milks in which acetic acid bacteria were present at only very low levels (e.g. Bel1, Ir2, UK1), or were not detected (e.g. Fr1, UK3), underwent a successful fermentation, as determined by a reduction in pH and milk coagulation after 24-48 hours (data not shown), it may be that acetic acid bacteria are not strictly required for the fermentation process but rather contribute in some other way. Our further studies will focus on elucidating the precise contribution of specific populations on the consistency of kefir milk. The fact that *Lachnospiraceae* and *Ruminococcaceae* are present in several grains but not detected in the milk samples implies a poor ability to proliferate in the milk medium. Bifidobacteria were detected in two grains only (Ir9, 0.81% and UK2, 0.10%). These findings, coupled with previous studies, establish that bifidobacteria represent only a minor proportion of the kefir grain consortium. Furthermore, its poor endurance in the kefir milk suggests that it would need to be added in an encapsulated, or other such form, if kefir were to be employed

as a vehicle for *Bifidobacterium* supplementation (Gonzalez-Sanchez et al., 2010). High-throughput sequencing also effectively unveiled the presence of a number of other rare populations in the kefir grains, which accounted for <1% of the overall population in most kefirs. Of these, *Faecalibacterium*, *Allistipes*, *Rickenellaceae*, *Allobaculum* and *Enterococcus* have not been identified in kefir previously and are typically associated with gut microbial populations. In contrast, *Pseudomonas* spp., identified in the grains of other high-throughput sequencing efforts in trace amounts, were not identified in these kefirs (Dobson et al., 2011, Leite et al., 2012).

After investigating the application of several ITS-specific databases, such as UNITE (http://unite.ut.ee/index.php), it was found that ITSoneDB, which consists of a comprehensive set of well-annotated and phylogenetically-classified ITS1 sequences derived form from Genbank and arranged on the NCBI taxonomy tree, gave the best assignment levels (Santamaria et al., 2012). The composition of the kefir-associated yeast population has been the subject of some attention (Farnworth, 2005, Lopitz-Otsoa et al., 2006) which has not been helped by nomenclature-related difficulties and a reliance, to date, on culture-based investigations. The *Saccharomycetaceae* have a poorly defined group-specific morphology and such a basis for classification can lead to unreliable distinction of species from close relatives. Furthermore, many yeasts of the *Ascomycota* and *Basidiomycota* have sexual (teleomorphic) and asexual (anamorphic) states of reproduction, sometimes leading to classification of species under two names. It has been proposed that in 2013, fungi shall be known by only their teleomorph name, unless in extenuating circumstances

(Hawksworth, 2011), and thus this approach has been taken here. Examination of the literature highlights that *Candida kefyr* has previously been shown to constitute up to 90% of the yeast population in kefir milk (Kwon et al., 2003) and has routinely been isolated from kefir (Witthuhn et al., 2005, Engel et al., 1986, Kwon et al., 2003). Despite a significant presence in the ITS database, no *Candida* were detected in this study. Notably, however, a number of reads which displayed similarity with *C. kefyr* were instead assigned to the corresponding teleomorph, *Kluyveromyces marxianus*, by virtue of higher percent similarity. *Kluyveromyces marxianus* has previously been associated with kefir (Latorre-Garcia et al., 2007, Gao et al., 2012, Loretan et al., 2003).

The dominant yeast detected in this study was *Kazachstania*, consisting of *Kazachstania barnetti* and *Kazachstania unispora. K*. *unispora* was previously known as *Saccharomyces unipsorus* (Kurtzman, 2003), which has been identified in kefir (Latorre-Garcia et al., 2007, Loretan et al., 2003, Zhou et al., 2009, Magalhaes et al., 2011, Gao et al., 2012) and has been associated with other fermented beverages (Montanari et al., 1996, Las Heras-Vazquez et al., 2003). It would appear that *K. unispora* is particularly well adapted to the dairy environment as it is the most prevalent species, out-competing rival species including *K. barnetti*. This marks the first time *K. barnetti*, found in the grain but not in the milk, has been identified in a kefir environment. *Naumovozyma* is a genus that closely resembles *Saccharomyces* and *Kazachstania*, and the species identified here, *Naumovozyma castellii*, was reclassified from *Saccharomyces castellii* in the past (Kurtzman and Robnett, 2003). Although it has not previously been linked with kefir, the only other species in the genus,

Naumovozyma dairenensis (formerly *Saccharomyces dairenensis*) has been (Martini and Kurtzman, 1988). In contrast to the significant presence of the aforementioned fungal species, the relative absence of *Saccharomyces* is at first striking given its historical association with kefir. This is most likely reflective of the reclassification of *Naumovozyma* and *Kazachstania*. Despite this, it is notable that previous studies have suggested that *Saccharomyces cerevisiae* is quite common in kefir (Gao et al., 2012, Motaghi et al., 1997, Simova et al., 2002) whereas here the genus was detected in just three grains and three milks, and in trace amounts. It is possible that this genus is not as widespread as previous evidence suggested or may have been misassigned in previous studies. Alternatively, *Saccharomyces* may be more common in kefirs from geographic locations not included in this study. The origin of the grain may also have been significant with respect to the identification of *Pichia kudriavzevii* (previously known as *Issatchenkia orientalis*) at levels that were atypically high, relative to other samples, in the Spanish kefir (grain, 0.57%; milk, 34.27%). Notably, Latorre-Garcia *et al* identified *Issatchenkia orientalis* as one of the most representative species of Spanish kefir (Latorre-Garcia et al., 2007) and, until recently (Gao et al., 2012), it had not been found among non-Spanish kefir grains or milks. With respect to other species, it was also notable that *Torulaspora delbreuckii* was not detected in this study despite the fact that both it (Angulo et al., 1993, Loretan et al., 2003) and its anamorph form, *Candida colliculosa*, have previously been detected in kefir (Wyder et al., 1997). There were also many instances whereby we identified species not previously detected in kefir milks, for instance while *Dekkera anomala* (anamorph:

Brettanomyces anomalus) has been isolated from kefir (Wyder et al., 1997), *Dekkera bruxellensis* (anamorph: *Brettanomyces bruxellensis*) has not been isolated from kefir before now (but has been found in traditional fermented Mongolean and Zimbabwean milks (Gadaga et al., 2001, Miyamoto et al., 2010)). Other species which had not previously been detected, but were present in lower abundance and few (often just one) milk sample(s) included *Cryptococcus* sp. *Vega* 039, *Zygosaccharomyces lentus*, *Penicillium* sp. *Vega 347*, *Wallemia sebi*, *Ganoderma lucidum*, *Cyberlindnera jadinii*, *Eurotium amstelodami*, *Heterobasidion annosum*, *Peziza campestris*, *Teratosphaeria knoxdaviesii*, *Dioszegia hungarica* and *Malassezia pachydermatis*. *Cryptococcus* and *Zygosaccharomyces* have been found in kefir before (Witthuhn et al., 2005), but this marks the first identification of the respective species, *Cryptococcus* sp. *Vega* 039 and *Z. lentus*. *Cryptococcus* is a ubiquitous basidiomycotic yeast that was previously identified in a kefir that had been frozen and recultivated. This point is noted as the *Cryptococcus*-associated milks described in the current study resulted from two kefir grains, Ir8 and Ir9, which had been recultivated from -80°C storage. *Z. lentus* is considered a food spoilage organism associated with low-pH beverages and can grow at low temperatures (Steels et al., 1999). *C. jadinii* is used in animal and human dietary supplements, and is a good source of vitamins, minerals, proteins and essential amino acids (Lee and Kim, 2001). Despite not being isolated from kefir, it has been used to scale-up single-cell protein production using kefir (Koutinas et al., 2005). Additionally, *E. amstelodami* is frequently isolated from bakers products (Abellana et al., 1999). *H. annosum*, *P. campestris*, *T. knoxdaviesii* and *D. hungarica* are all regarded as

environmental fungi. *H. annosum* is the causative agent in the root and butt rot of pine trees (Asiegbu et al., 2005), *Peziza* is associated with saprophytic cup fungal growth on rotten wood (Hansen et al., 2005), *Teratosphaeria* have been described as eucalyptus pathogens (Hunter et al., 2009) and *D. hungarica* has been shown to inhabit arbuscular mycorrhizal fungi (Renker et al., 2004). *M. pachydermatis*, detected in Ir9, is a known pathogen which threatens neonatal infants and has been associated with domesticated canines (Chang et al., 1998). Finally, in multiple samples (Bel1, Fr1, Ger1, Ger2, It1 and UK1-5), many *Saccharomycetaceae*-associated reads could not be assigned at the genus level and were designated as "other" (**Figure 4**). It is anticipated that as more fungal sequences are deposited, the species corresponding to these reads can be uncovered. The PCoA plots visualising the kefir microfloras do not show any obvious clustering amongst the different kefirs, showing the diversity between kefir populations regardless of their source.

The fact that natural kefir is capable of hosting several health-associated organisms suggests it could theoretically be altered to incorporate pre-established and certified probiotic strains, with minimal sensory impact. Indeed, the ultimate application of kefir may be as a potential delivery system for viable health-promoting organisms to the gut (Ivanova et al., 2012). However, the fact that grains have yet to be produced from pure culture (Chen et al., 2009a) suggests that there remains a lot to be understood regarding the population dynamics of kefir grains.

In conclusion, the study represents the most comprehensive investigation of the microbial composition of kefir grains and milks to date. It

provides important information that may facilitate the reconstitution of kefir grains to create tailored kefir grains and milks while further investigation of the specific components identified can reveal their contribution to the kefir grain structure and the health-promoting aspect of the associated beverages.

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CHAPTER V

Sequence-based analysis of the microbial composition of water kefir from multiple sources

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5.1 ABSTRACT

Water kefir is a water-sucrose based beverage, fermented by a symbiosis of bacteria and yeast to produce a final product that is lightly carbonated, acidic and that has a low alcohol percentage. The microbes present in water kefir are introduced via water kefir grains, which consist of a polysaccharide matrix in which the microorganisms are embedded. We aimed to provide a comprehensive sequencing-based analysis of the bacterial population of water kefir beverages and grains, while providing an initial insight into the corresponding fungal population. To facilitate this objective, four water kefirs were sourced from the UK, Canada and the United States. Culture-independent, high-throughput, sequencing-based analyses revealed that the bacterial fraction of each water kefir and grain was dominated by *Zymomonas*, an ethanol-producing bacterium, which has not previously been detected at such a scale. The other genera detected were representatives of the lactic acid bacteria and acetic acid bacteria. Our analysis of the fungal component established that it was comprised of the genera *Dekkera*, *Hanseniaspora*, *Saccharomyces*, *Zygosaccharomyces*, *Torulaspora* and *Lachancea*. This information will assist in the ultimate identification of the microorganisms responsible for the health-promoting attributes of these beverages.

5.2 INTRODUCTION

Water kefir is a water-sucrose beverage that is fermented to produce carbon dioxide and low concentrations of ethanol. Traditionally, figs or other dried fruit and lemon are added to provide additional minerals and flavour, with acidity being proportional to the length of the fermentation process. Water kefir is fermented via a combination of bacteria and yeast which live in symbiosis in water kefir grains. Also known as Tibicos or sugary kefir, water kefir differs from the more popular milk-based kefir in that the grains are principally composed of dextran, and are translucent and crystal-like in appearance (Neve and Heller, 2002).

There are two prevalent theories as to the origin of water kefir, but the exact source remains unknown. The first theory is that soldiers returning from the Crimean war brought grains to western Europe (Ward, 1892), while the second proposes the spontaneous formation of grains on the pads of the *Opuntia* cactus in Mexico (Lutz, 1899). Regardless, water kefir grains were passed from household to household and this is still the most common means by which grains and beverages are acquired as water kefir production has yet to be commercialised on a significant scale.

Water kefir is less studied than other fermented beverages such as milk kefir or kombucha and, as such, any claimed health benefits have yet to be confirmed. Culture-based studies have shown that the bacterial component is comprised of a varied mixture of *Lactobacillus*, *Lactococcus, Leuconostoc* and *Acetobacter* (Pidoux, 1989, Pidoux et al., 1990, Franzetti et al., 1998, Neve and Heller, 2002, Gulitz et al., 2011). Culture-independent methods have also been

used to assess the microbial populations of water kefir, including one recent study that used high-throughput sequencing to assess the bacterial component of four water kefir grains (Gulitz et al., 2013). The yeast component has most commonly been identified, *via* culture-based studies, as *Saccharomyces*, *Hanseniaspora*/*Kloeckera*, *Zygotorulaspora* and *Candida* (Pidoux, 1989, Neve and Heller, 2002, Waldherr et al., 2010, Gulitz et al., 2011). It is estimated that bacteria are present in the grains at approx 10^6-10^8 per gram, with yeast numbers at 10^6-10^7 per gram (Gulitz et al., 2011).

As a potential functional/health-promoting food, it is noteworthy that water kefir contains strains from species with which health benefits have been frequently attributed, such as lactobacilli, bifidobacteria (Gulitz et al., 2013) and to a lesser extent, *Saccharomyces*-related yeasts (Czerucka et al., 2007, Desreumaux et al., 2011, Foligne et al., 2010). Water kefir therefore represents a potentially effective means of probiotic delivery. With this in mind, the aim of this study was to characterise the bacterial and, to a lesser extent, yeast components within water kefir using high-throughput, culture-independent techniques. This study represents the first occasion upon which high-throughput sequencing has been utilised to investigate the fungal component of water kefir, and together with the corresponding bacterial analysis, provides a comprehensive insight into the microbiological profile of water kefir. It is worth noting that this study reports the microbial composition of water kefir grains composed of dextrose, which have not been associated with milk fermentation, unlike a small number of studies reporting water kefir that would appear to

have been prepared from milk kefir grains introduced into sucrose-water solution (Puerari et al., 2012, Miguel et al., 2011).

5.3 MATERIALS AND METHODS

5.3.1 Culture maintenance

Four water kefir grains were acquired from commercial and individual suppliers from Canada [Ca], the UK [UK] and the United States [US1 and US2]. The cultures were fermented under uniform conditions by adding 60g grains/litre of sterilised Ballygowan® mineral water supplemented with 10% sucrose, followed by the addition of one dried, organic fig (Rainbow Organic Wholefoods, Ireland). The culture was fermented at 25°C and after 24 hours DNA was extracted from the fermentate and the grains. Each culture was routinely changed two times per week for a minimum of 10 fermentation cycles prior to extraction and cultures were kept separate from one another to prevent cross-contamination.

5.3.2 Metagenomic DNA extraction

To extract DNA from the fermentate, 1.8mls was centrifuged to generate a pellet which was suspended in 450 µl of lysis buffer P1 from the Powerfood Microbial DNA Isolation kit (MoBio Laboratories Inc, USA). The resuspended pellet was subjected to enzymatic digestion with enzymes mutanolysin (100 U/ml; Sigma) and lysozyme (50 µg/ml; Sigma) at 37°C for 1 hour, followed by proteinase K (250 µg/ml; Sigma) digestion at 55°C for 1 hour. Extraction was optimised with a 10 minute 70°C incubation (Quigley et al., 2012) prior to mechanical lysis. The Powerfood Microbial DNA Isolation kit was then used as

per the manufacturer's instructions. For extraction of DNA from the grain, 1g of grains were washed twice in sterile H_2O and homogenised using a mortar and pestle, after which DNA was isolated using a modified phenol-chloroform-based extraction procedure (Garbers et al., 2004).

5.3.3 DNA amplification and pyrosequencing

PCR amplification of the V4-V5 variable region (408 bp) of the 16S rRNA gene was performed using the universal primers F1 (5'-AYTGGGYDTAAAGNG) and R5 reverse (5'-CCGTCAATTYYTTTRAGTTT) to allow an investigation of the bacterial component of the microbial populations (Claesson et al., 2010). Unique multiplex identifier adaptors were attached between the 454 adaptor sequences and the forward primers. Tagged universal primers were also used to amplify fungal DNA from the variable ITS-1 rRNA region (Buee et al., 2009). In this instance the forward primer ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA) and ITS2 reverse (5'-GCTGCGTTCTTCATCGATGC) generated two different sets of PCR products of *circa* 410 bp and 250 bp. To prevent preferential sequencing of the smaller-sized reads, these were pooled and sequenced separately. Amplicons generated from three PCR reactions of template DNA were pooled and cleaned using the Agencourt AMPure® purification system (Beckman Coulter; Takeley, UK). Sequencing of the 16S rRNA V4-V5 and ITS1 rDNA ribosomal amplicons was performed using a 454 Genome Sequencer FLX (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) at Teagasc Food Research Centre, Moorepark.

5.3.4 Pyrosequencing data analysis

Raw sequences were quality trimmed and filtered using the Qiime Suite of programs (Caporaso et al., 2010). Any reads not meeting a minimum quality score of 25 and sequence length shorter than 150 bps for 16S amplicon reads and 200 bps for ITS amplicon reads were discarded. Trimmed fasta sequences were assessed by BLAST analysis against the SILVA database (version 100) for 16S reads and a previously published ITS-specific database for ITS samples (Pruesse et al., 2007, Santamaria et al., 2012). BLAST outputs were parsed using MEGAN (Huson et al., 2007); a bit-score of 86 was employed for 16S ribosomal sequence data, and a bit-score of 35 was used for ITS sequence data. Reads were deposited in the SRA database under the accession number ERP002661.

5.4 RESULTS AND DISCUSSION

5.4.1 High-throughput sequencing reveals the alpha and beta diversity of the bacterial population in water kefir

In this study, both grains and fermentates were analysed, as it has been shown that the grains and fermentates of associated beverages can differ (e.g. milk kefir), with the solid material typically having a more complex microbial population than the final fermented beverage (Marsh et al., 2013). To prevent an origin-based bias, water kefir was sourced from different geographical locations, and was fermented to simulate natural artisanal fermenting conditions. DNA was extracted from both the grain and 24-hour fermentate to afford the most accurate assessment of total microbial population.

Amplicons corresponding to the V4-V5 variable region of the bacterial 16S rRNA gene were generated using universal tagged primers. To determine species richness and diversity, Chao1 values and Shannon and Simpson indices were calculated, in addition to Phylogenetic Diversity and Observed Species numbers (**Table S1**). Values for these were low by comparison to other environmental analyses, highlighting low alpha diversity. Rarefaction curves were calculated at 97% similarity, and are approaching parallel to the x-axis for all samples, indicating sufficient reads were obtained to adequately assess the bacterial population (**Figure S1**). Beta diversity was measured using the unweighted UniFrac distance matrix. A Principal Coordinate Analysis (PCoA) plot was generated based on unweighted UniFrac distance matrices in order to visualise any clustering amongst the different groups (**Figure S2**). No clustering was evident based on sample type or origin, but it was apparent that the data points corresponding to grains of US2 and Ca were each distantly removed from all other data points establishing a quite different beta diversity among these samples. ITS rDNA amplicons were in general sequenced at a lower depth and thus corresponding alpha and beta diversity analyses were not completed.

5.4.2 The bacterial component of water kefir was dominated by the genus *Zymomonas*, with a LAB and AAB presence

16S reads from both the grain and the fermentate were assigned to three bacterial phyla; Actinobacteria, Firmicutes and Proteobacteria. Across both the grains and fermentates, the dominant phyla were Proteobacteria and Firmicutes. Proportions of Proteobacteria were in general greater in the grain

(>70%) than the fermentates (50-60% abundance for Ca, UK and US1 but 75% abundance for US2; **Table S2**). Proportions of Firmicutes were consistently higher in the fermentates than in the grains. The abundance of Actinobacteria was low in general but was higher in each of the grains than in the fermentates.

The most striking difference between a recent high-throughput sequencing-based study (Gulitz et al., 2013) and data presented here relates to the fact that in this study the most abundant genus was *Zymomonas*. Indeed, *Zymomonas* was dominant in all grains and fermentates, with proportions being consistently higher in the grains. Proportions of *Zymomonas* ranged from 87% in the grain of US1 to 49.5% in the fermentate (**Figure 1**). *Zymomonas* is traditionally regarded as a fermentation-associated microorganism and forms part of the microbial population of several fermented beverages from plants in tropical regions of America, Africa and Asia. It has been isolated from Mexican pulque, a fermented beverage made from agave sap which resembles water kefir if theories with respect to the *Opuntia* cactus are correct (Ward, 1892). Additionally, it has already been established that *Zymomonas* can constitute part of the microbial population of water kefir (Hsieh et al., 2012), albeit not at similarly high levels.

Zymomonas is capable of producing ethanol in such high quantities that it rivals the fermentation capabilities of *Saccharomyces* (Panesar et al., 2006). *Zymomonas* species have also been shown to produce levan, a polysaccharide used as a thickening agent, which has been demonstrated to have antitumor effects (Calazans et al., 1997, Yoon et al., 2004), immunostimulating activity (Xu et al., 2006), effects on lipid metabolism (Yamamoto et al., 1999) and prebiotic

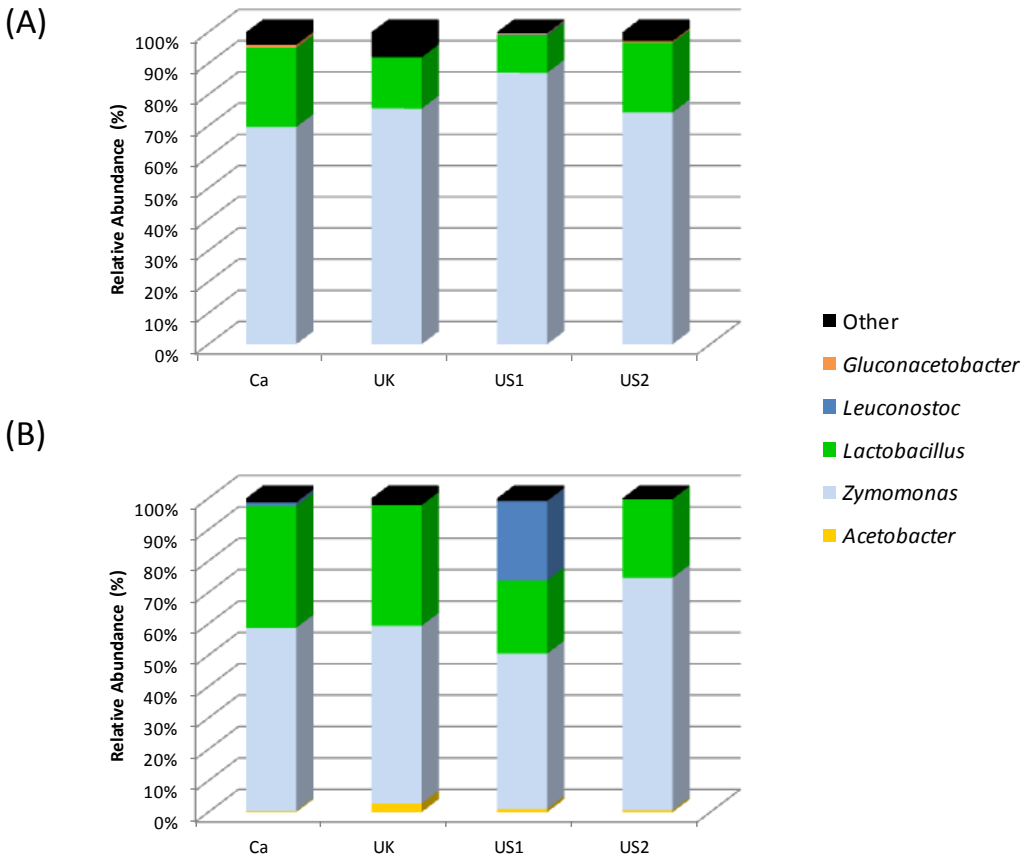

Figure 1

16S phylogenetic composition of the bacterial component of the water kefir grain (A) and kefir fermentate (B) at genus level.

activity (Dal Bello et al., 2001, Jang et al., 2003). *Zymomonas* itself has been shown to be safe for consumption (de Azeredo et al., 2010) and to have beneficial immunomodulatory effects by protecting against sepsis in mice (Campos et al., 2013). Further analysis is required to characterise the strains of *Zymomonas* present in these beverages and their contribution, through the production of leaven, sorbitol and ethanol, on the consistency, flavour and safety of the final beverage. Nonetheless, at the significant proportions detected, members of this genus are undoubtedly of considerable importance with respect to the composition of these beverages. It should be noted that while *Zymomonas* represents a dominant component of these water kefirs, additional studies with a larger number of samples will be required to assess the distribution of this genus in beverages from other regions and prepared in different ways.

The majority of non-*Zymomonas* reads from the water kefir grains and fermentates were assigned to the lactic acid bacteria (LAB), and *Lactobacillus* in particular. This genus has long been associated with water kefir and in a number of instances has been reported to be the dominant bacterial genus present (Gulitz et al., 2013, Gulitz et al., 2011). Here *Lactobacillus* accounted for 25.4%, 16.4%, 12% and 22.3% of the grains of Ca, UK, US1 and US2, respectively, and 38.8%, 38.5%, 23.4% and 25.1% of the corresponding fermentates, respectively, and thus proportions increased from the grain to the fermentate in all instances. *Lactobacillus hilgardii* was believed to be the primary exopolysaccharide producer in water kefir (Pidoux, 1989, Waldherr et al., 2010), whereas Gulitz *et al* reported *L. nagelii* and *L. hordei* to be the major EPS

producers (Gulitz et al., 2011). *Leuconostoc* was recognized as the major LAB in the fermentate of US1 (25.6%), but was less abundant in the grain environment (Ca, 1.1%; US1, 0.4%). Thus, while water kefir-associated *Leuconostoc* has been shown to be competitive and to be capable of EPS-production (Gulitz et al., 2011), its detection in such low grain abundance suggests that this genus does not contribute significantly to grain formation in these samples. No lactococci were detected in our study, despite representatives of this genus having been isolated as part of culture-based studies of other water kefirs previously (Pidoux, 1989, Waldherr et al., 2010).

The genera *Acetobacter* and *Gluconacetobacter* were represented as a minor proportion of the overall bacterial component of water kefir (**Figure 1**). *Gluconacetobacter* were present in the grains of Ca, US1 and US2 at <1%, and were undetected in the fermentates and UK grain, whereas, interestingly, *Acetobacter* appeared to be better adapted to the fermentate (Ca, 0.4%; UK, 2.8%; US1, 1.1% and US2, 0.6%). Acetic acid bacteria have been consistently detected at trace levels in water kefir studies (Franzetti et al., 1998, Gulitz et al., 2011, Gulitz et al., 2013) but, as yet, no role has been attributed to them.

Members of the *Bifidobacteriaceae* family were detected at low proportions, but could not be confidently assigned to genus level. The presence of *Bifidobacterium* in water kefir was only recently revealed via high-throughput sequencing (Gulitz et al., 2013), but had not been detected by previous culture- or molecular-based analyses. Our analysis showed levels of *Bifidobacteriaceae* were lower than in the fermentate (<2%) than the grain (Ca, 3.6%; UK, 7.8%;

US1, 0.2% and US2, 2.5%), perhaps indicative of poor survival of representatives from this family in a non-EPS protected environment.

5.4.3 Insight into the fungal composition of water kefir and associated water kefir grain

As the primary focus of this study was the investigation of the bacterial composition of kefir grains and beverages, fungal (ITS) amplicons were sequenced at a much lower depth with a view to providing an initial insight into this population. Amplification with ITS primers yielded two amplicons of approximately 250 bp and 410 bp, respectively, which were purified and sequenced separately. The existence of two pools limited, to a certain extent, inter-pool comparisons but the dominant components of the fungal population were nonetheless evident. All reads were assigned to the phylum Ascomycota. At the family level *Saccharomycetaceae* and *Saccharomycodaceae* were detected in both the grain and water kefir fermentate, while *Debaromycetaceae* were detected in the fermentate only (**Table S3**). Almost all reads from the 250 bp pool were assigned to the genus *Dekkera* while the 410 bp pool contained amplicons corresponding to a variety of species.

Dekkera was represented by two species, *Dekkera anomala* and *Dekkera bruxellensis*. Both are common fermentation-associated species, with *D. bruxellensis* frequently associated with the spoilage of fermented beverages (Heresztyn, 1986). To our knowledge, *Dekkera* has not been previously identified in water kefir but has been found in other fermented beverages (Pintado et al., 1996, Wyder et al., 1997, Miyamoto et al., 2010). The fact that

Dekkera has previously been overlooked by culture-based approaches may in part be due to the fact that it has a slow doubling time when cultured on standard yeast media and, thus, can be outcompeted by other genera such as *Saccharomyces* (Ibeas et al., 1996).

From the 410 bp pool, proportions of *Saccharomyces* were highest in the fermentate of US1. *Saccharomyces* were also detected in the fermentates of Ca and US2, despite having not been detected in the corresponding grains (**Table S3**). *Saccharomyces* could only be assigned to the species level among ITS reads corresponding to the UK grain, with 1.3% of reads being assigned as *Saccharomyces cerevisiae*. This has previously been identified as a key yeast in several water kefirs (Franzetti et al., 1998, Gulitz et al., 2011). Despite this, the proportions of *Saccharomyces* detected in our study were lower than expected. Waldherr *et al* also noted this phenomenon, which suggests that levels of *Saccharomyces* may have been overestimated in previous studies (Waldherr et al., 2010). The genus *Hanseniaspora*, whose anamorphic form is *Kloeckera*, has frequently been detected in water kefir, most often as *Hanseniaspora valbyensis* and *Hanseniaspora vineae* (Galli et al., 1995, Franzetti et al., 1998, Waldherr et al., 2010, Gulitz et al., 2011). Both species were also detected in our study. While *H. vineae* was detected in more samples, it was generally (with the exception of US1) present at a lower abundance than *H. valbyensis*. *H. valbyensis* was most prominent in the Ca fermentate, and was also present in the US1 fermentate and UK grain. The genus *Lachancea* was not detected in the fermentate, but was found in the grain of UK, with all reads being assigned to *Lachancea fermentati*. This species was formerly known as *Zygosaccharomyces*

fermentati (Kurtzman, 2003) and has been detected in water kefir previously (Gulitz et al., 2011). *Torulaspora* was detected in the grain of UK and the fermentates of US1 and US2, but could not be assigned at the species level. *Torulaspora* has been identified in water kefir on only one previous occasion as *Torulaspora pretoriensis* (Pidoux, 1989). *Zygotorulaspora florentina* (formerly *Zygosaccharomyces florentinus*/*Saccharomyces florentinus* (Kurtzman, 2003)) was unexpectedly not identified in any of the cultures, despite frequently appearing as a dominant species in previous analyses (Gulitz et al., 2011, Neve and Heller, 2002, Pidoux, 1989). While it is possible that it was present in lower abundance and thus was below our detection limits, this species probably does not exert the positive impact on the growth of lactobacilli that that has been attributed to it in previous studies (Leroi and Pidoux, 1993a, Stadie et al., 2013, Leroi and Pidoux, 1993b). It may be that another species of yeast performs this role within the samples assessed on this occasion.

Finally, two other yeast have been detected which have not previously been associated with water kefir. One species of *Zygosaccharomyces*, *Zygosaccharomyces lentus*, was detected at low levels in the fermentate of UK and grains of Ca, US1 and US2. *Z. lentus* is often considered a spoilage yeast (Steels et al., 1999) and this is the first report of the detection of this species in water kefir. *Meyerozyma* was detected in the fermentates of US1 and US2, but of these reads only 0.3% from US2 could be assigned at the species level (from the 250 bp pool; **Table S4**) to *Meyerozyma caribbica*.

In conclusion, we have established that the bacterial population of a number of water kefirs analysed through high-throughput sequencing consists

of a dominant population of *Zymomonas* with lactic acid bacteria (*Lactobacillus*, *Leuconostoc*), acetic acid bacteria (*Acetobacter* and *Gluconacetobacter*) and *Bifidobacteriaceae* being detected in descending proportions. While dextran-producing lactic acid bacteria are not the dominant bacteria, it would seem that the possibility exists for the modulation of the microbiology of water kefir through the introduction of non-indigenous lactobacilli and bifidobacteria to facilitate its use as a non dairy-based system for the delivery of probiotics. Our results revealed that the yeast component of the water kefir samples was comprised of several species previously associated with water-kefir, but notably a number of species not traditionally associated with water kefir, including *Dekkera*, *Zygosaccharomyces* and *Meyerozyma* were also identified.

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CHAPTER VI

Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples

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6.1 ABSTRACT

Kombucha is a sweetened tea beverage that, as a consequence of fermentation, contains ethanol, carbon dioxide, a high concentration of acid (gluconic, acetic and lactic) as well as a number of other metabolites and is thought to contain a number of health-promoting components. The sucrose-tea solution is fermented by a symbiosis of bacteria and yeast embedded within a cellulosic pellicle, which forms a floating mat in the tea, and generates a new layer with each successful fermentation. The specific identity of the microbial populations present has been the focus of attention but, to date, the majority of studies have relied on culture-based analyses. To gain a more comprehensive insight into the kombucha microbiota we have carried out the first culture-independent, high-throughput sequencing analysis of the bacterial and fungal populations of 5 distinct pellicles as well as the resultant fermented kombucha at two time points. Following the analysis it was established that the major bacterial genus present was *Gluconacetobacter*, present at >85% in most samples, with only trace populations of *Acetobacter* detected (<2%). A prominent *Lactobacillus* population was also identified (up to 30%), with a number of sub-dominant genera, not previously associated with kombucha, also being revealed. The yeast populations were found to be dominated by *Zygosaccharomyces* at >95% in the fermented beverage, with a greater fungal diversity present in the cellulosic pellicle, including numerous species not identified in kombucha previously. Ultimately, this study represents the most accurate description of the microbiology of kombucha to date.

6.2 INTRODUCTION

Kombucha is a sweetened, black tea beverage, which is fermented to contain ethanol and carbon dioxide. Traditionally fermented for 8-10 days, kombucha has a sour taste, resembling that of sparkling apple cider, which develops over prolonged fermentation into a mild vinegar flavour (Blanc, 1996, Reiss, 1994). The tea is fermented by the presence of a cellulosic pellicle or mat that rests above the broth forming a fresh layer with each successful fermentation. The fermentation itself is performed by a symbiosis of bacteria and yeast embedded within the cellulosic matrix. Kombucha, known by several names, including tea fungus and Haipao (Liu et al., 1996), has been brewed in China for over 2000 years, where it was fermented by many households. Although probiotic effects have yet to be directly associated with kombucha-derived microorganisms (Kozyrovska et al., 2012), initial studies have shown promising health benefits in relation to the tea itself. Such health benefits include anti-carcinogenic (Jayabalan et al., 2011) and anti-diabetic (Hiremath et al., 2002, Aloulou et al., 2012) effects, treatment for gastric ulcers (Banerjee et al., 2010) and high cholesterol (Yang et al., 2009), and it also has been shown to impact immune response (Ram et al., 2000) and liver detoxification (Loncar et al., 2000).

The majority of microbiology-orientated studies of kombucha to date have been culture-based. These are limited in that certain species can be difficult to isolate and the exclusive reliance on phenotypic traits can lead to misidentification (Raspor and Goranovic, 2008). Additionally, culture-based studies tend to be low-throughput and thus only a certain proportion of isolates will ever be investigated. Traditionally, only a few genera of bacteria have been

isolated from kombucha, most frequently *Acetobacter* (Hesseltine, 1965, Sievers et al., 1996, Liu et al., 1996, El-Salam, 2012, Dutta and Gachhui, 2006, Zhang et al., 2011, Chen and Liu, 2000), but species of *Gluconacetobacter* and *Lactobacillus* have also been identified (Zhang et al., 2011, Wu et al., 2004, Trovatti et al., 2011, Yamada et al., 1997, Yang et al., 2010). The most important of these species are those which produce bacterial cellulose, such as *Komagataeibacter xylinus*, which was recently reclassified from *Gluconacetobacter xylinus* (Yamada et al., 2012) (also previously known as *Acetobacter xylinum* (Yamada et al., 1997)) and is considered the best studied and most efficient bacterial cellulose producer in kombucha (Mikkelsen et al., 2009, Strap et al., 2011). The yeast population is generally believed to be more varied in composition (Mayser et al., 1995, Teoh et al., 2004). Yeasts identified include species of the genera *Zygosaccharomyces*, *Candida*, *Kloeckera/Hanseniaspora*, *Torulaspora*, *Pichia*, *Brettanomyces*/*Dekkera*, *Saccharomyces* and *Saccharomycoides* (Teoh et al., 2004, Liu et al., 1996, Mayser et al., 1995, Hesseltine, 1965, Jankovic and Stojanovic, 1994, Chen and Liu, 2000, Markov et al., 2001). The role of yeasts in the fermentation of kombucha is to convert sucrose to organic acids, carbon dioxide and ethanol, with the latter then used by acetic acid bacteria to form acetaldehyde and acetic acid. Acetic acid bacteria also use yeast-derived glucose to synthesise bacterial cellulose and gluconic acid (Dufresne and Farnworth, 2000, Greenwalt et al., 1998). Bacterial and yeast numbers are generally thought to reach 10^4 – 10^6 cfu ml^{-1} ml in the kombucha after approximately 10 days of fermentation, with yeast slightly outnumbering bacteria (Teoh et al., 2004, Chen and Liu, 2000,

Goh et al., 2012). Microbial counts have been reported to be greater in the tea broth than the cellulosic pellicle (Goh et al., 2012). It has also been found that the viability of the microbes present decreases gradually over the course of the fermentation due to oxygen starvation and extremely acidic (pH 2.5) conditions (Chen and Liu, 2000). While first generation (Sanger) sequencing of 16S rRNA genes has been used to identify kombucha bacterial isolates (El-Salam, 2012, Trovatti et al., 2011, Dutta and Gachhui, 2006), the significant developments in the field of microbial ecology that have been possible through the use of high-throughput, culture-independent techniques (Ercolini, 2013) have, to date, yet to be applied to kombucha populations.

In this study, high-throughput amplicon sequencing was performed on DNA extracts from cellulosic pellicles sourced from 5 distinct geographic locations and from the corresponding kombuchas at two time points during fermentation. This analysis provides the most in-depth analysis of the kombucha microflora to date.

6.3 MATERIALS AND METHODS

6.3.1 Culture maintenance

Five kombucha cellulose pellicles with approximately 200 ml starter culture were acquired from suppliers from different geographic locations. Two kombucha samples were sourced from Canada and designated Ca1 and Ca2; other kombucha samples were sourced from the United Kingdom (UK), United States (US) and Ireland (Ire). All kombuchas were cultivated under uniform conditions. 2L of tap water was sterilised at 121°C for 15mins in a 3L glass container. The water was brought to the boil and 0.49% w/v black tea (Barry's Original Blend) was added, and allowed to steep for 15 mins. After removal of the tea leaves, 10% sucrose was added and stirred to dissolve. Once the sucrose-tea solution had cooled to room temperature, 10% fermented tea (of the previous fermentation brew from kombucha with the same origin, corresponding to the aforementioned starter culture) was added to acidify the solution. The cellulose pellicle was placed in the culture, light side up. The container was covered with a 100% cotton towel and fixed with an elastic band. Cultures were fermented at room temperature (23°C) and re-inoculated into fresh tea every 10 days. Samples were taken at days 3 and 10 of fermentation for DNA extraction.

6.3.2 Metagenomic DNA extraction

To extract DNA from the fermented kombucha at day 3 and 10 of fermentation, 1.8ml of fermented tea was centrifuged to generate a pellet which was suspended in 450µl of lysis buffer P1 from the Powerfood Microbial DNA

Isolation kit (MoBio Laboratories Inc, USA). The resuspended pellet was subjected to enzymatic digestion with enzymes mutanolysin (100U/ml) and lysozyme (50µg/ml) at 37°C for 1 hour, followed by proteinase K (250µg/ml) digestion at 55°C for 1 hour. Extraction was optimised with a 10 minute 70°C incubation (Quigley et al., 2012) prior to mechanical lysis using the Qiagen TissueLyser II (Retsch®). The Powerfood Microbial DNA Isolation kit was then used as per the manufacturer's instructions. Pure DNA was eluted in HPLC-grade sterile water. For extraction of DNA from the pellicle, 0.25g of cellulosic pellicle was removed from the surface mat of a fresh fermentation, washed twice in sterile H_2O, and chopped into small fragments using a sterile blade. 0.3g of sterile glass beads and 750µl of cellulase (Sigma-Aldrich) were added to a microcentrifuge tube containing the pellicle, which was mechanically lysed for 10mins in a Qiagen TissueLyser II (Retsch®). The solution was incubated for 1 hour at 40°C, after which it was centrifuged to generate a pellet. The supernatant was discarded and the pellet was resuspended in 450µl of pre-warmed buffer P1. The extraction was then subjected to enzyme digestion and the modified Powerfood extraction was performed as described above.

6.3.3 DNA amplification and high-throughput sequencing

Metagenomic DNA extracts were used as a template for PCR amplification. PCR amplification of the V4-V5 variable region (408 bp) of the 16S rRNA gene was performed using the universal primers F1 (5'-AYTGGGYDTAAAGNG) and R5 reverse (5'-CCGTCAATTYYTTTRAGTTT) to facilitate an investigation of the

bacterial component of the microbial populations (Claesson et al., 2010). Unique multiplex identifier adaptors were attached between the 454 adaptor sequences and the forward primers to facilitate the pooling and subsequent differentiation of samples. Tagged universal primers were also used to amplify fungal DNA from the variable internal transcribed spacer (ITS)-1 rDNA region (Buee et al., 2009). In this instance the forward primer ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA) and ITS2 reverse (5'-GCTGCGTTCTTCATCGATGC) generated two different sets of PCR products of *circa* 410 bp and 250 bp. To prevent preferential sequencing of the smaller amplicons, the bands were extracted separately using the Roche High Pure PCR Cleanup Micro Kit, and two pools were created and sequenced separately. The PCR conditions used for 16S amplification were 94°C denaturation for 2 min, 35 cycles of 94°C for 1 min (denaturation), 52°C for 1 min (annealing) and 72°C for 1 min (extension) followed by a final 72°C for 2 mins. The PCR conditions used for ITS amplification were 94°C denaturation for 4 min, 35 cycles of 94°C for 30 seconds (denaturation), 50°C for 1 min (annealing), and 72°C for 1 min and 30 seconds (extension). A final annealing step of 72°C for 10 mins was performed. All DNA was subject to a 10 min hotstart at 94°C prior to PCR amplification. Amplicons generated from three PCR reactions/template DNA were pooled and cleaned using the Agencourt AMPure® purification system (Beckman Coulter Genomics, Takeley, UK). Purified products were quantified using the Nanodrop 3300 Fluorospectrometer (Thermo Scientific) and the Quant-iT™ Picogreen® dsDNA Assay kit (Invitrogen). Equal concentrations of 16S or ITS amplicons were

pooled, AMPure cleaned and assessed by an Agilent 2100 Bioanalyser (Agilent Technologies) to determine purity and to ensure the absence of primer dimers. Sequencing of the 16S rRNA V4-V5 and ITS-1 rDNA ribosomal amplicons was performed using a 454 Genome Sequencer FLX Titanium System (Roche Diagnostics Ltd) at Teagasc Food Research Centre, Moorepark, according to 454 protocols.

6.3.4 Amplicon sequencing data analysis

Raw sequences were quality trimmed and filtered using the Qiime Suite of programs (Caporaso et al., 2010); any reads not meeting the quality criteria of a minimum quality score of 25 and sequence length shorter than 150 bps for 16S amplicon reads and 200 bps for ITS amplicon reads were discarded. The maximum homopolymer limit was increased to 10 for ITS amplicons as ITS sequences are known to harbour long homopolymer runs. All chimeras were removed using ChimeraSlayer (http://microbiomeutil.sourceforge.net/#A_CS). Trimmed fasta sequences were assessed by BLAST analysis against the SILVA database (version 100) for 16S reads and a previously published ITS-specific database for ITS samples (Pruesse et al., 2007, Santamaria et al., 2012). MEGAN was used to parse BLAST outputs (Huson et al., 2007) and a bit-score of 86 was employed for 16S ribosomal sequence data, with a bit-score of 35 for ITS sequence data. The QIIME suite of programs was used to calculate alpha diversity including Choa1 richness, Shannon diversity, Simpson index, Phylogenetic Diversity and Observed species (Caporaso et al., 2010). Sequences were clustered into operational taxonomical units, chimera checked (0.11% of

16S and 0.02% of ITS sequences removed) and aligned using the default pipeline within Qiime for 16S sequences and a modification to implement MUSCLE (Edgar, 2004) for the alignment of ITS sequences. The FastTree package (Price et al., 2009) was used to generate phylogenetic trees for both sequence types. For 16S sequences, default clustering and alignment methods were implemented within Qiime, whilst the default pipeline was modified for the ITS sequences. Sequencing depth was estimated using rarefaction analysis. QIIME was also used to generate weighted UniFrac, unweighted UniFrac and Bray-Curtis distances matrices. Principal Co-ordinate Analysis plots based on these distance matrices were generated with Qiime and visualised using King software (Chen et al., 2009). Reads were deposited in the SRA database under the accession number ERP002661.

6.4 RESULTS AND DISCUSSION

6.4.1 High-throughput sequencing reveals the α and β diversity of the microbial population in kombucha

In advance of carrying out this study, a number of steps were taken to ensure that the results generated would be as representative as possible. Five kombuchas were acquired from individual and commercial suppliers from Canada ([Ca1] and [Ca2]), Ireland [Ire], the United Kingdom [UK] and the United States [US], in order to mitigate against geographical bias. Although a sample number of five kombuchas will not provide a definitive microbial analysis, this is the first time high-throughput, culture-independent analysis has been applied to the microbial population of kombucha and, as such, represents the most in-depth study to date. As it has been proposed that the microbiological composition of the fermented product can vary with time (Teoh et al., 2004, Sreeramulu et al., 2000), DNA was extracted from the fermented kombucha tea (fermentate) at days 3 and 10 of the fermentation and from the pellicle. Given that the pH of the fermented product dropped as the process continued, reaching pH 3-3.5 by day 10, the microorganisms detected at this time point reflect a more acid tolerant population. It was also decided that the sucrose-tea solution should not be sterilised by autoclaving in advance of inoculation as this has in the past been shown to generate toxic chemicals inhibitive of kombucha growth (Teoh et al., 2004). Instead, culturing conditions adhered to were those closest to that which occur in the home, with black tea and sucrose considered the best substrates for metabolic and microbial activity (Kallel et al., 2012).

Using universal tagged primers, amplicons corresponding to the V4-V5 region of the bacterial 16S rRNA gene were generated. This region was chosen as it provides longer reads which enable more confident assignments than shorter products generated by amplification of other variable regions. Following high-throughput sequencing of the amplicons, a total of 15,209, 13,409 and 13,400 reads were successfully assigned to the pellicle, day 3 and day 10 extracts, respectively, equating to a respective average of 3,042, 2,682 and 2,680 reads per individual sample (**Table S1**). To identify fungal populations present, ITS rDNA amplicons were generated from the variable ITS-1 region using primers adapted from Buee *et al* (Buee et al., 2009). These primers produced amplicons of *circa* 410 bps in all samples. Additional amplicons of *circa* 250 bps from UK (the pellicle, day 3 and day 10) were also sequenced. In order to prevent preferential sequencing of the smaller-sized reads, the respective amplicons were extracted individually via gel extraction and separated into two pools according to approximate size, and sequenced independently, with the quality-approved reads subsequently combined to form an overall total. Due to the approach taken, comparisons between ITS amplicons of specific sizes, rather than of all amplicons, was carried out. It was found that reads from the 250 bp pool were assigned specifically to the genus *Dekkera* whereas amplicons from other fungi were approx. 400 bp in length. In total, 11,795 reads were obtained for the pellicle, corresponding to an average of 2,949 reads per individual sample. Total reads for day 3 and day 10 were 18,592 and 37,868, respectively (**Table S1**).

To determine species richness and diversity, Chao1 values, Shannon and Simpson indices, Phylogenetic Diversity and Observed Species were calculated (**Tables S2-S3**). The values for each were reflective of the naturally low bacterial and fungal diversity in fermented beverages (Marsh et al., 2013) relative to other environmental samples such as gastrointestinal or soil populations. Rarefaction curves, calculated at 97% similarity, are approaching parallel to the x-axis for all samples, indicating sufficient reads were obtained to adequately assess the populations (**Figure S1**). Averages for Observed Species and Phylogenetic Diversity suggest that bacterial diversity was greatest at day 3 of the fermentation. Across all measures of alpha diversity, except Chao1, diversity was shown to be greater within the fungal component of the kombucha population than the bacteria.

Beta diversity was measured using three different distance matrices i.e. weighted UniFrac, unweighted UniFrac and Bray-Curtis (weighted UniFrac and Bray-Curtis data not shown). Visualisation of the populations, reflecting the different samples and time points, was performed by generating Principal Co-ordinate Analysis (PCoA) plots (**Figure 1**). From this analysis it was shown that the bacterial populations of the pellicles were distinct from one another, as evidenced by a lack of clustering. However, there was some clustering amongst day 3 samples, and by day 10, the bacteria had converged on a more comparable population, denoted by clustering in blue. Thus the pellicle

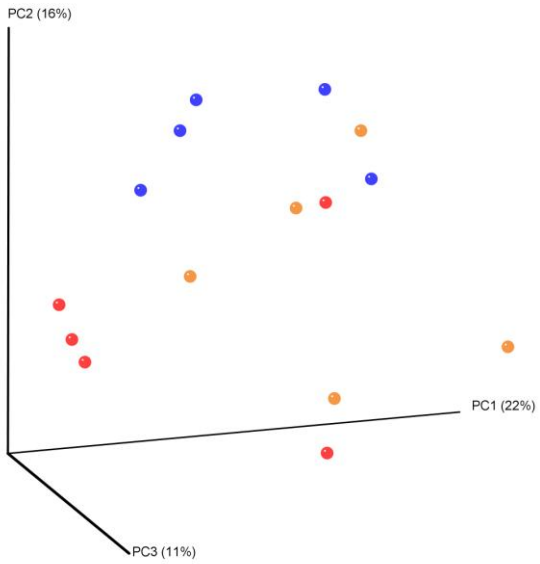

Figure 1 Principle Coordinate Analysis (PCoA) plots, based on unweighted UniFrac distance matrices, show clustering of the bacterial populations from several kombuchas, where Red = day 3 extracts, Blue = day 10 extracts and Orange = pellicle extracts

populations were shown to possess the greatest diversity, with only the most competitively fit populations dominating within the fermented medium.

6.4.2 16S analysis reveals a dominant *Gluconacetobacter*, with a prominent LAB, presence in kombucha

Five bacterial phyla were identified across the various samples, including Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes and Proteobacteria. Of these, Proteobacteria were the most abundant, accounting for >90% of reads in each sample, with the exception of Irish-sourced kombucha, where this phylum constituted 87.9% of the kombucha population at day 3 of the fermentation and 60% of the pellicle (**Table S4**). Members of this phylum consisted of 2 genera, *Acetobacter* and *Gluconacetobacter*. In every kombucha, and in every sample type, reads assigned to *Gluconacetobacter* greatly exceeded those of *Acetobacter* (**Figure 2**; **Table 1**). Indeed, *Gluconacetobacter* accounted for >85% of assigned reads from all samples, with the exception of Ire, where it is present at a low of 58% in the pellicle, making it the dominant bacterial genus in every sample. The genus *Acetobacter* was consistently found in Irish-sourced kombucha only, where proportions were highest in the pellicle (1.9%) but represented <1% of the bacterial population in the fermentates. Although 16S reads of this length cannot be accurately assigned beyond genus level due to the high level of sequence homology, the reclassification of *Acetobacter xylinum* to *Gluconacetobacter xylinus* (Yamada et al., 1997), a species considered to be the dominating bacterium and principal

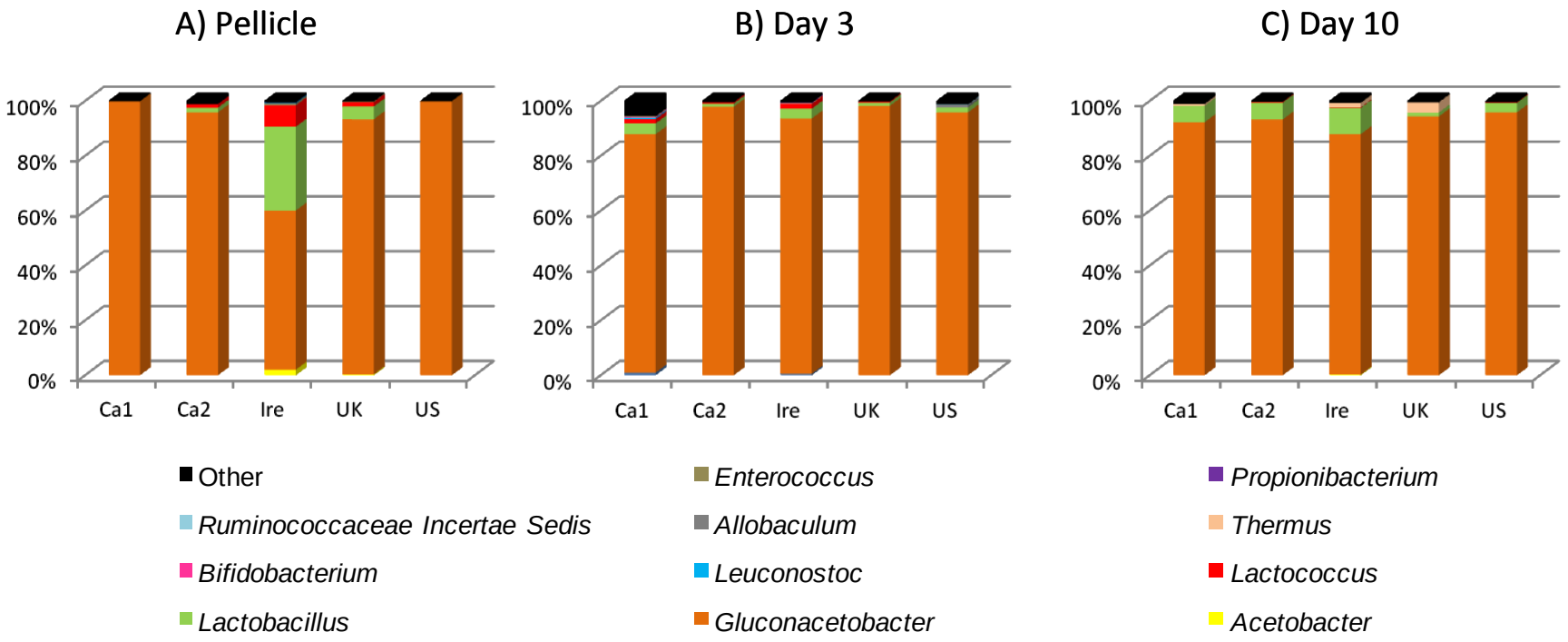

Figure 2 16S phylogenetic composition of the bacterial component of the kombucha pellicle (A) and the tea at day 3 (B) and day 10 (C) of fermentation, at genus level.

Day 3	Ca1	Ca2	Ire	UK	US
Acetobacter	0.86	0	0.43	0	0
Gluconacetobacter	86.91	97.79	93.09	98.14	95.73
Lactobacillus	3.93	1.13	3.57	1.19	1.77
Lactococcus	1.56	0.51	1.77	0.37	0
Leuconostoc	0.52	0	0	0	0
Bifidobacterium	0.3	0	0	0	0
Thermus	0.22	0	0.2	0	0
Allobaculum	0	0	0	0	0.88
Ruminococcaceae Incertae Sedis	0	0	0	0	0.19
Propionibacterium	0	0	0.2	0	0
Other	5.75	0.58	0.75	0.3	1.42

Day 10	Ca1	Ca2	Ire	UK	US
Acetobacter	0	0	0.19	0	0
Gluconacetobacter	92.17	93.16	87.62	94.26	95.73
Lactobacillus	5.96	6.17	9.59	1.44	3.47
Lactococcus	0	0.23	0.19	0	0.18
Leuconostoc	0	0	0	0	0
Bifidobacterium	0	0	0	0	0
Thermus	0.66	0	1.62	3.73	0
Allobaculum	0	0	0	0	0
Ruminococcaceae Incertae Sedis	0	0	0	0	0
Other	1.22	0.45	0.79	0.58	0.61

Pellicle	Ca1	Ca2	Ire	UK	US
Acetobacter	0	0	1.93	0.28	0
Gluconacetobacter	99.77	95.73	58.02	92.97	99.82
Lactobacillus	0	1.72	30.57	4.64	0
Lactococcus	0	1.29	7.76	1.62	0
Leuconostoc	0	0	0	0	0
Bifidobacterium	0	0	0	0	0
Thermus	0	0	0	0	0
Allobaculum	0	0	0	0	0
Ruminococcaceae Incertae Sedis	0	0	0	0	0
Enterococcus	0	0	0.69	0.21	0
Propionibacterium	0	0	0.38	0	0
Other	0.23	1.26	0.66	0.28	0.18

Table 1

Relative abundances of the 16S bacterial genera at day 3, day 10 and in the pellicle.

contributor of bacterial cellulose in kombucha (Hesseltine, 1965, Sievers et al., 1996, Mikkelsen et al., 2009, Strap et al., 2011), might partially account for the high frequency of the genus *Gluconacetobacter*, and the relative lack of *Acetobacter*. Moreover, the genus *Gluconacetobacter* has recently been subdivided into three genera: *Nguyenibacter*, *Komagataeibacter* and *Gluconacetobacter* (Vu et al., 2013, Yamada et al., 2012). Most noteworthy is the fact that *G. xylinus* is now the type strain of the genus *Komagataeibacter*, and is known as *Komagataeibacter xylinus*. It is possible that reads assigned to *Gluconacetobacter* may actually belong to the genus *Komagataeibacter* as, at the time of writing, microbial databases have yet to be updated to accommodate the reclassification. In addition, while there are risks associated with assigning short 16S amplicons at the species level, it was noted here that the most prominent *Gluconacetobacter* hits were to an uncultured species and *Gluconacetobacter* sp. Rh1-MS-CO.

More recently, there have also been several other species of *Komagataeibacter* and *Gluconacetobacter* identified in kombucha including *Komagataeibacter kombuchae* (Dutta and Gachhui, 2007), *Gluconacetobacter* sp A4 (Yang et al., 2010) and *Gluconacetobacter sacchari* (Trovatti et al., 2011). The high proportions of *Gluconacetobacter* also indicate that other species of *Acetobacter*, such as *A. aceti* (Liu et al., 1996, El-Salam, 2012), its close relative *A*. *nitrogenificans* (Dutta and Gachhui, 2006), *A. pasteurianus* (Chen and Liu, 2000, Liu et al., 1996) and *A*. *liquefaciens* (Zhang et al., 2011) that have been found in the past to be a dominant component in kombucha are not as common in the kombuchas prepared in this study or were inaccurately classified in the

past (Sreeramulu et al., 2000). Similar acetic acid species have been found in related wine and vinegar cultures (Gonzalez et al., 2004, De Vero et al., 2006).

The detection of considerable proportions of lactic acid bacteria (LAB), such as *Lactobacillus* and *Lactococcus*, in kombucha pellicles is another key observation. Reads representative of the corresponding phylum, the Firmicutes, were consistently detected in the fermentates, increasing in each instance from day 3 to 10, and were also detected in the pellicles of Ca2, Ire and UK at abundances of 3.3%, 39.4% and 6.6%, respectively. Of the fermented teas, the Irish kombucha contained the greatest proportions of Firmicutes at 5.6% and 10.5% in the day 3 and day 10 extracts, respectively. The Firmicutes-associated reads were predominantly assigned to the genus *Lactobacillus*, which was detected in all fermentates, but only in the pellicles of Ca2, Ire and UK. Proportions of lactobacilli were lowest in the day 3 extracts, with proportions being higher at day 10 in all instances. Lactobacilli have been isolated in only two previous kombucha studies, both of which focused on Chinese kombuchas (Zhang et al., 2011, Wu et al., 2004), and thus the frequency and abundance at which lactic acid bacteria were detected in this study is particularly novel. The Irish-sourced pellicle stood out in this regard with >35% of reads assigned at genus level corresponding to lactic acid bacteria. The data presented here suggests that lactobacilli are more prevalent in kombucha than was previously understood, particularly at the latter stages of fermentation. Studies have indicated that one role for these microorganisms might be to assist in the growth of acetic acid species in that *Lactobacillus* has been shown to greatly increase cellulose production by *Gluconacetoabcter* in co-culture (Seto et al.,

2006) and, even more relevantly, LAB have been shown to support the growth of *Gluconacetobacter* in kombucha (Yang et al., 2010). The abundance of *Lactococcus* varied but was generally lower, consistently representing <2% of reads across all fermentates and pellicles with the exception of the Irish pellicle (7.8%). Proportions were greatest in the three pellicles in which it was detected, and numbers reduced from <2% in four of the five day 3 fermentates to <1% in three day 10 fermentates, suggesting poor lactococcal survival as the fermentation proceeded. It appeared that the most frequent species of *Lactobacillus* identified was *Lactobacillus kefiranofaciens* subsp. *kefirgranum*. A number of other genera from the Firmicutes were detected at low proportions, which have not been identified in kombucha previously, including *Leuconostoc* (Ca1, day 3 - 0.5%), *Allobaculum* (US, day 3 - 0.9%), *Ruminococcaceae Incertae Sedis* (US, day 3 - 0.3%) and *Enterococcus* (Ire and UK, pellicle – 0.7% and 0.2% respectively) (**Table 1**). The number of different genera detected was greatest among the day 3 samples.

Actinobacteria were less common amongst the kombuchas, with reads corresponding to the genera *Propionibacterium* (Ire, day 3 - 0.2%; pellicle - 0.4%) and *Bifidobacterium* (day 3 - 0.3%). To our knowledge this marks the first time that *Bifidobacterium* has been identified in natural kombucha, though it should be noted that there are instances of it having been added to commercially brewed kefir. The low abundance of both bifidobacteria and propionibacteria, and their absence from day 10 samples, suggests that these bacteria do not thrive in the fermented environment. It should be noted that the presence of these traditionally gut-associated organisms may reflect their

introduction through poor sanitation or some other means at some point during the formation of specific pellicles and thus there is no guarantee that these populations are active in this environment. However, in the case of the Actinobacteria, it's worth mentioning that these microorganisms have been detected at similar abundances in fermented milk (Dobson et al., 2011), and bifidobacteria can constitute a significant proportion of the microbiota of fermented water beverages (Gulitz et al., 2013). Further investigations will be required to ultimately determine the source of individual populations and their contribution to the formation and health-promoting characteristics of kombucha. This point also relates to other populations detected at low frequencies. Reads corresponding to the phylum Deinococcus-Thermus were only consistently detected across Ca1 samples (0.2%, 0.9% and 0.2% at day 3, day 10 and pellicle, respectively), but were also present in the fermented Irish (day 3 - 0.2%; day 10 1.6%) and UK (day 10 - 3.8%) teas. Reads from this phylum were assigned to the genus *Thermus*. The presence of bacteria corresponding to this genus is interesting, since these species are traditionally regarded as being associated with thermophillic niches (Vajna et al., 2012, Beffa et al., 1996). The specific requirements for culturing such bacteria, in this case, high temperature, might explain why these and other such species have not been detected through culture-based approaches previously. Reads corresponding to the phylum Bacteroidetes were found in the US sample only at day 3 of the fermentation at 0.4% abundance. Reads that could not be assigned at the phylum level consistently represented <1% across all samples, and were not assigned to the level of genus. Finally, it was notable that a number of 16S reads

corresponding to the order Sphingomonadales and the family *Lachnospiraceae*, respectively, could not be further assigned. Again, this marks the first occasion upon which these microorganisms have been identified in kombucha, though *Sphingobium* and *Zymomonas*, genera of Sphingomonadales, have been identified in beverages fermented by milk and water kefir grains respectively (Ninane et al., 2007, Hsieh et al., 2012).

6.4.3 The fungal population in kombucha is dominated by *Zygosaccharomyces* with a variable sub-dominant population

From a fungal perspective, the use of culture-independent approaches was particularly beneficial as the difficulties associated with slow growth on yeast-specific media makes culture-based assessments difficult. The only fungal phylum detected in the fermented tea was Ascomycota, while Basidiomycota, the other phylum of the sub-kingdom Dikarya, was also detected in the cellulose pellicle extracts of Ca1, Ca2, and UK at sub-dominant levels.

Zygosaccharomyces was the dominant genus in all pellicle and broth extracts from Irish and Canadian-sourced kefirs (**Figure 3**; **Table 2**), accounting for >95% of each population with the exception of the pellicle of Ca1, which contained a lower abundance (79%) as a consequence of containing a higher proportion of *Pichia* (8.3%) and unassigned (10.9%) reads. Genera proportions of the fermented teas matched that of the corresponding pellicle, thereby suggesting that the fungal composition of the cellulosic pellicle used to inoculate the tea is the key determinant.

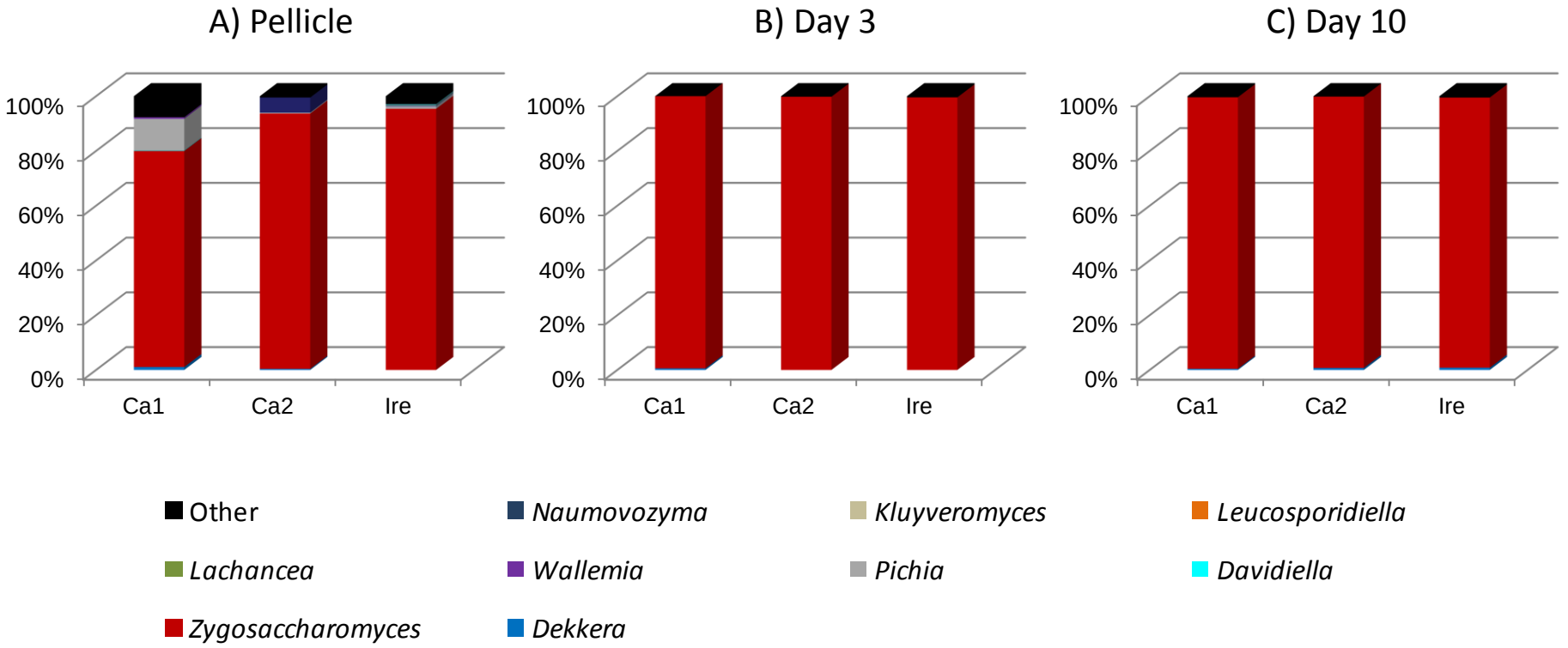

Figure 3 ITS phylogenetic composition of the fungal component of the kombucha mat (A) and fermented tea at day 3 (B) and day 10 (C) of fermentation, at genus level, and the kombucha mat (D) and fermented tea at day 3 (E) and day 10 (F) of fermentation, at species level.

Day 3	Ca1	Ca2	Ire1
Dekkera	0.57	0	0
Zygosaccharomyces	99.43	99.8	99.57
Kazachstania	0	0	0
Other	0	0.2	0.43

Day 10	Ca1	Ca2	Ire1
Dekkera	0.43	0.74	0.83
Zygosaccharomyces	99.19	99.13	98.69
Kazachstania	0	0	0
Other	0.38	0.14	0.49

Pellicle	Ca1	Ca2	Ire1
Dekkera	1.07	0.41	0
Zygosaccharomyces	78.96	93.26	95.39
Davidiella	0.16	0	0
Pichia	11.54	0	0.62
Wallemia	0.58	0	0
Lachancea	0	0.3	0
Leucosporidiella	0	5.56	0
Kazachstania	0	0	0.33
Kluyveromyces	0	0	0.25
Naumovozyma	0	0	0.54
Meyerozyma	0	0	0
Saccharomyces	0	0	0
Hanseniaspora	0	0	0
Other	7.68	0.48	2.87

Table 2

Relative abundances of the ITS fungal genera at day 3, day 10 and in the pellicle.

The occurrence of amplicons of *circa* 250 bp in the pellicle, day 3 and day 10 extracts of UK-sourced kombucha necessitated sequencing of these smaller-sized reads separately from the regular 400 bp pool. Most reads from this pool were assigned to the genus *Dekkera* (**Table S5**). Mayser *et al*. have previously found that *Brettanomyces* (the anamorph of *Dekkera*) and *Zygosaccahromyces* were the most common yeast genera in a culture-based study of German kombucha samples (Mayser et al., 1995). There have also been some other instances upon which both *Zygosaccharomyces* and *Dekkera* have been identified (Teoh et al., 2004, Liu et al., 1996).

Analysis of ITS sequence data also facilitated assignment at the species level. The *Zygosaccharomyces* population consisted of two species, *Zygosaccharomyces lentus* and *Zygosaccharomyes bisporus*, with *Z. lentus* present in consistently higher abundance, and in the case of Irish-sourced kombucha, was the only species detected. It has been proposed that *Z. lentus* isolates have probably been phenotypically misidentified as *Z. bailii* in the past (Steels et al., 1999a), which may account for the reported presence of *Z. bailii* in some culture-based studies (Teoh et al., 2004, Liu et al., 1996, Chen and Liu, 2000). The dominant *Dekkera* species in UK samples was *Dekkera bruxellensis*, which out-numbered the *Dekkera anomala,* present in the pellicle. The identification of this particular species is consistent with some previous studies (Liu et al., 1996, Teoh et al., 2004). *Dekkera bruxellensis*, often considered a spoilage yeast, is commonly found in fermented beverages such as fruit juices, red wine, beer and cider where it is responsible for the development of unpleasant odour and taste (Heresztyn, 1986b, Heresztyn, 1986a). Conversely, it

is thought to have a beneficial contribution to the flavour of lambic beer (DeKeersmaecker, 1996). Similarly, *Zygosaccharomyces* sp. are problematic in the brewing and juice industries with *Z. lentus* in particular being considered a food spoilage yeast due to its osmotolerance, ability to grow at low pH, and resistance to preservatives (Steels et al., 1999b). *Kazachstania* was the only other genus detected among the tea broths, being present in UK day 10, and is solely represented by the genus *Kazachstania unispora* which has not been linked with kombucha previously. Considering the variation in kombucha-associated yeast, it is likely that the role of yeast in kombucha fermentation can be performed by several non-specific fermentative yeast, with *Zygosacharomyces*-dominated populations being particularly common. Investigation of fermentations produced by defined starters is required to elucidate the exact contribution of each yeast to the final flavour and biochemical composition of kombucha.

While *Zygosaccharomyces*, *Dekkera* and *Kazachstania* were the only genera detected across the various fermented tea samples, several other genera were detected in the pellicle samples and were represented by one species. Of these, *Davidiella tassiana*, *Lachancea fermentati*, *Kluyveromyces marxianus*, *Naumovozyma castelli* and the Basidiomycota representatives, *Wallemia sebi* and *Leucosporidiella fragaria*, have not been found in kombucha previously. Of the others, *Hanseniaspora* has previously been identified in one kombucha study (Mayser et al., 1995) while *Pichia kudriavzevii* and other *Pichia* and *Pichia*-like species such as *P. fermentans* and *P. membranaefaciens* and *Hyphopichia burtonii*, have been detected on a number of previous occasions

(Mayser et al., 1995, Jankovic and Stojanovic, 1994, Chen and Liu, 2000). Although there were a number of hits that could not be assigned to a species within the fungal database, it is believed that, as more sequences are deposited, it will be possible to assign greater proportions of fungal ITS sequences from studies such as this one.

Ultimately, it would appear that the naturally low pH and ethanol content of the beverage generated under regular, household brewing conditions, combined with other forms of competition involving the indigenous microbial population, is sufficient to limit contamination from undesirable populations. This indigenous population appears robust, with those microbes contained within the matrix of the cellulosic pellicle dictating the microbiota of the eventual beverage. Further investigations will reveal the link between the different populations, their bioactive peptides and the purported health benefits of kombucha. It is believed that the information presented here will assist in future microbiology-focused kombucha studies and may ultimately assist in the development of defined kombucha starter cultures.

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CHAPTER VII

Screening of milk kefir, water kefir and kombucha for antimicrobial producers

7.1 ABSTRACT

A high-throughput screening strategy was employed to identify antimicrobial producers among microbes cultured from the fermented beverages kefir, water kefir and kombucha. In addition to identifying producers of the lactococcin ABM bacteriocin complex from the milks of three Irish kefirs, a proteinaceous antimicrobial was found to be produced by the yeast *Dekkera bruxellensis*, isolated from kombucha sourced from both Ireland and the United States. This antimicrobial was consistently active against strains of *Lactobacillus bulgaricus* but not other targets. Characterisation of the protein showed it to be active in a pH range of 3-8, moderately heat stable, negatively charged and have a mass >30 kDa. On the basis of these characteristics the novel antimicrobial is thought to be a glycosylated mycocin.

7.2 INTRODUCTION

Interest in functional foods has increased greatly in recent years, with functional beverages becoming particularly popular amongst consumers (Granato et al., 2010). Several attributes of such beverages can account for their description as 'functional', including their nutritional, organic and probiotic properties. There are a number of traits that contribute to the probiotic potential of a microorganism, one being the production of antimicrobial peptides known as bacteriocins (Dobson et al., 2012, Gillor et al., 2008). These peptides are produced by bacteria and are active against other, typically closely-related, bacteria. Producers are immune to their own bacteriocin via self-immunity mechanisms, and are mainly produced by gram positive bacteria (Arnison et al., 2013, Nes et al., 1996). Bacteriocins from food-grade bacteria have the potential to be used as effective, natural additives in lieu of chemical food-preservatives, while bacteriocin-producers can also inhibit undesirable microorganisms when introduced as starter cultures (Galvez et al., 2007, Cleveland et al., 2001). Additionally, given the shortage of novel antibiotic discoveries to combat emerging pathogen resistance, the potential use of bacteriocins for therapeutic applications has been the focus of ever greater attention (Cotter et al., 2013). Yeast are also capable of producing proteinaceous antimicrobials, known as mycocins or yeast killer toxins, which are analogous to bacteriocins (Magliani et al., 1997). Producers are referred to as killer yeast, and the genes responsible for killer toxins can be encoded by several different genomes including dsRNA viral particles that reside

symbiotically within yeast cells, chromosomal DNA, and linear DNA plasmids. These killer proteins are larger than bacteriocins, ranging in size from 10-300 kDa, often contain glycosyl residues and are typically active at acidic pHs (**Table 1**). As with bacteriocins, killer toxins are typically most active against closely related species and producers are immune to their own toxins. Killer toxins have several potential applications as antifungals in human therapy and agriculture as well as in the prevention of wine spoilage and biotyping (Meinhardt and Klassen, 2009). A study by Comitini *et al* showed that protease- and heat-sensitive antimicrobials, distinct from mycocins, were produced by *Saccharomyces cerevisiae* and displayed activity against *Oenococcus oeni*, a wine bacterium (Comitini et al., 2005). While detailed characterisation of these peptides was not completed, analysis indicated a complex mix of proteinaceous yeast metabolites contributed to the inhibitory activity.

The aim of this study was to screen the microbial populations of the fermented beverages kefir, kombucha and water kefir for the presence of antimicrobial producers. In the past, fermented foods have proven to be a good source of bacteriocin-producers. This is consistent with the observation that environments with a high cell-density of competing/complex microbial populations have been shown to harbour bacteriocin producers in the past (Kreth et al., 2006, Riley and Gordon, 1999). Fermented foods from which bacteriocin producers have been sourced include, for example, kimchi (Han et al., 2013, Choi et al., 1999), kefir (Powell et al., 2007, Atanassova et al., 1999, Ryan et al., 1996, Powell, 2006), fermented sausage (Alvarez-Cisneros et al.,

2010, Rekhif et al., 1995), sauerkraut (Harris et al., 1992, Ge et al., 2009) and cheese (Izquierdo et al., 2009, Ghrairi et al., 2005). The beverages selected for

Table 1: A list of known mycocins, their producers and toxin sizes (Adapted from Meinhardt & Klassen 2009)

Organism	Killer Toxin	Toxin Size (kDa)
Chromosomal DNA		
Pichia anomala NCYC 434	K5/Panomycin	49
Pichia anomala ATCC 96603	PaKT/PKT	85
Pichia anomala DBVPG 3003	Pikt	>3
Pichia farinosa KK1	SMKT	α 6.6 / β 7.9
Pichia membranfaciens CYC1106	PMKT	18
Williopsis californica DSM 12865	Wicaltin	34
Williopsis saturnus IFO 0117	HYI	9.5
Kluyveromyces wickerhamii DBVPG 6077	Kwkt	>10
Kluyveromyces phaffii DBVPG 6076	KpKt	33
Kluyveromyces amrxianus NCYC 587	K6	42
Saccharomyces cerevisiae 111	KHR	20
Saccharomyces cerevisiae 115	KHS	75
Extrachromosomal dsRNA		
Saccharomyces cerevisiae KL88	K1	α 9.5 / β 9.0
Saccharomyces cerevisiae CBS8112	K28	α 10 / β 11
Hanseniaspora uvarum 470	N/A	18
Zygosaccharomyces bailii 412	Zygocin	10
Extrachromosomal dsDNA		
Kluyveromyces lactis IFO1267	Zymocin	α 99.0 / β 30.0 / γ 28.0
Pichia inositovora NRRL Y-18709	N/A	>100
Pichia acaciae NRRL Y-18665	PaT	α 110.0 / β 39.0 / γ 38.0
Debaromyces robertsiae CBS6693	N/A	>100

this study are fermented by a mixed population of bacteria and yeast at room temperature, and are protected from spoilage by undesirable microorganisms, suggesting the presence of additional inhibitory mechanisms. Kefir is a milk-based beverage and originates from the Caucasian mountains, whereas water kefir is fermented sucrose-water and is believed to derive from the pads of the Opuntia cactus in Mexico. Both beverages are fermented by polysaccharide grains harbouring a symbiosis of bacteria and yeast. The bacterial component is typically composed of *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc* and some species of acetic acid bacteria (Marsh et al., 2013c, Marsh et al., 2013b). Kombucha is a sweetened tea of Chinese origins, fermented by acetic acid bacteria, some lactobacilli and a variety of yeast (Marsh et al., 2013a). The populations of these beverages are known to be influenced by external factors such as the environment and country of origin, and can vary considerably from one culture to another.

With recent technological advances, high-throughput screening is increasingly being employed for bacteriocin discovery. High-throughput robotics can be used to screen banks of bioengineered bacteriocin producers for isolates with enhanced activity (Field et al., 2008), while screening of environmental samples for specific phenotypes lead to the discovery of planosporicin and microbisporicin (Castiglione et al., 2007, Castiglione et al., 2008). Screening has also been used in the isolation of bacteriocin-producing lactic acid bacteria from malt samples (Rouse et al., 2007, Marsh et al., 2012). Using similar high-throughput robotics, the focus of this study was to create a bank of bacterial

isolates from the aforementioned fermented beverages, to screen these isolates for antimicrobial production and to characterise associated antimicrobial peptides/proteins.

7.3 MATERIALS AND METHODS

7.3.1 *Maintenance of fermented cultures*

Kefir grains were sourced from different countries including 9 grains from Ireland (designated IR1, 2, 3, 4, 5, 6, 8, 9 and 10), 5 from the UK (UK1-5), 4 from the United States (designated US1, 2, 3 and 5) as well as others from Spain (Sp1), France (Fr1), Italy (It1), Canada (Ca1) and Germany (Ger1 and Ger2) (Marsh et al., 2013c). Kefirs were fermented in 10% reconstituted skimmed milk (RSM), which had been sterilized at 115°C for 15mins. Water kefir grains were sourced from Canada [Ca], the UK [UK] and the United States [US1 and US2] and fermented by adding 60g grains/litre of sterilised Ballygowan® mineral water supplemented with 10% sucrose, followed by the addition of one dried, organic fig (Rainbow Organic Wholefoods, Ireland) (Marsh et al., 2013b). Kombucha samples sourced from UK (UK), Ireland (Ire), Canada (Ca1 and Ca2) and the United States (US) and were prepared in black tea with 10% sucrose and 10% previous fermentate to acidify the culture (Marsh et al., 2013a). All cultures were fermented at room temp (23-25°C).

7.3.2 *Strains and growth conditions*

Listeria innocua DPC 3572, *Salmonella* Typhimurium LT2 and *Staphylococcus aureus* ATCC 25923 were cultured in Brain-Heart Infusion (BHI) at 37°C (Oxoid).

Lactobacillus bulgaricus DPC 5838 was grown in MRS (Difco) anaerobically at 37°C. *Leuconostoc mesenteroides* subsp. cremoris DPC 223 was cultured in MRS at 30°C while *Lactococcus lactis* MG 1363 and a pMRC01^{+} derivative were both cultivated on M17 (Difco) supplemented with 0.5% glucose (Sigma) at 30°C. Other bacteria used as indicators are listed in **Table 2**. All yeast indicators (**Table 3**) were grown on potato

dextrose agar (PDA) (Neogen, USA) at 25°C for 5 days, and maintained on PDA slants at 4°C. Overnights were prepared by inoculating the culture into potato dextrose broth and growing at 25°C for 48-72 hours.

7.3.3 *Sample preparation*

1g of kefir grain and 1g of kombucha cellulose pellicle were rinsed twice in sterile water and blended separately using a T-25 Digital Ultra-Turrax in 9mls Ringers solution (Sigma) until homogenised. 1g of rinsed water kefir grains were homogenised using a sterile mortar and pestle and added to 9mls of Ringers. Microbes from kefir and water kefir were isolated after an initial 24 hour fermentation, and microbes from kombucha were isolated after 10 days of fermentation. All samples were serially diluted in Ringers to give approx 1000 CFUs per Qtray (Molecular Devices). Qtrays were prepared by pouring 250mls of the relevant molten agar, supplemented with 1% nystatin (Sigma), into a sterile Qtray and allowed to set on a flat surface. 1ml of the diluted sample was spread across the surface using sterilised 5mm borosilicate beads (Sigma). The media employed were: Wallerstein Laboratory (WL) fermentation media (Oxoid) (to select for specific kombucha isolates), while GM17 and MRS were used to select

for presumptive lactococci and lactobacilli from all sources. These agars were incubated aerobically at 30°C and anaerobically at 37°C, respectively. Tryptic soy agar, supplemented with yeast extract (TSA-YE), was also used as a non-selective nutritious medium to culture microbes from a variety of sources and was incubated at 37°C. Cultures were grown for 24-48 hours.

7.3.4 *Robotic screening*

The QPix2-XT (Genetix, New Milton, Hampshire, UK) was used to pick single colonies from the plates, which were then added to 384-well plates (Molecular Devices) containing freezing buffers. These buffers were prepared by adding K_2HPO_4 (36 mM), KH_2PO_4 (13.2 mM), sodium citrate (1.7 mM), $MgSO_4$ (0.4 mM), $(NH_4)2SO_4$ (6.8 mM) and 4.4% glycerol to the relevant growth media. Isolates were incubated appropriately overnight and stored at -80°C. 6 x 384-well plates were stamped over the surface of each Qtray containing the appropriate media and allowed to grow for up to 48 hours (**Figure 1**). Plates were then exposed to UV light for 30 minutes to inactivate the colonies, which were then overlaid with molten sloppy agar (0.75%) containing 0.25% of indicator bacterium. After further overnight incubation the plates were inspected for zones of inhibition. In instances where a strain merited further inspection, it was sourced from the corresponding frozen 384-well stocks. In specific instances where pH buffering of agar was required, 3-(N-morpholino)propanesulfonic acid (MOPS) was added at 0.2M or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 0.2M.

7.3.5 *Antimicrobial activity assays*

Antimicrobial activity against bacteria was assayed using the well diffusion method. Briefly, 20mls of agar seeded with 50ul of indicator strain was allowed to set, holes were bored in the agar, and 50ul of cell free supernatant (CFS) from an overnight culture of the putative antimicrobial producer was added to the wells. Plates were incubated overnight and visually inspected to identify zones of inhibition.

Antimicrobial activity against yeast was assessed using three methods. In the first two cases, overnight cultures of the yeast indicators were counted using a hemocytometer and cells were diluted to seed PDA with 10^5cells/ml. For the first assay, the putative antimicrobial producer was streaked across the surface of the seeded agar. In the second, wells were made and 50ul CFS from an overnight culture of the putative antimicrobial producer was added. Finally, yeast versus yeast antagonism was also tested using a swab approach, where 10^6 cells were swabbed across the surface of both a regular and NaCl-enriched (4%) agar plates, and Whatman discs soaked in crude antimicrobial protein preparation were placed on the surface (Golubev and Tomashevskaya, 2012, Llorente et al., 1997). All plates were incubated at 25°C and monitored over 5 days.

7.3.6 *Species identification*

DNA was extracted from overnight cultures of strains of interest according to the protocol of the Roche High Pure PCR Template Preparation Kit (Roche). PCR

amplification of the bacterial 16S gene was carried out using the universal 16S primers CO1 AGTTTGATCCTGGCTCAG and CO2 TACCTTGTTACGACTT. For fungi, PCR amplification of the internal transcribed spacer (ITS-1) was carried out using primers ITSF CTTGGTCATTTAGAGGAAGTAA and ITSR GCTGCGTTCTTCATCGATGC. DNA was quantified and amplicons were sent to Source Bioscience (Dublin, Ireland) for Sanger sequencing and speciation. Species were identified through BLAST analysis of the resultant DNA sequences.

7.3.7 *pH, temperature, solvent and protease stability*

The stability of antimicrobial-containing CFS following exposure to a variety of treatments was assessed. The pH of the CFS was measured using a pH meter (Hannah Instruments) and adjusted to a range of pH 2-9 using dilute HCl and NaOH to assess the pH range over which the antimicrobials were active. The sensitivity of the antimicrobial-containing CFS to protease was tested through treatment with equal volumes of the following enzymes: proteinase K (25mg/ml), alpha-chymotrypsin (10mgs/ml), trypsin (10mg/ml) and pepsin (25mg/ml). To assess temperature stability, samples were heat treated at 60°C, 70°C, 80°C and 90°C for 15mins each. To determine the sensitivity of the supernatants to solvents, 300ul of supernatant was combined with 700ul ethanol, acetonitrile, methanol, and isopropanol, respectively, and additionally with the same solvents containing 0.1% trifluoroacetic acid (TFA), and allowed to equilibrate for 6 hours prior to assaying. Following treatments the level of antimicrobial activity retained was assessed via well diffusion using *L. lactis* HP and *L*. *bulgaricus* DPC 5838, as indicators.

7.3.8 *Protein characterisation*

To determine the charge of the peptides and proteins and, in turn, decide how best to proceed with their purification, individual samples were passed through several columns including C18 Solid Phase Extraction (SPE), SCX-SPE and the anionic resins SAX (Phenomenex), and DEAE and Q Sepharose (GE Healthcare). Passage through an Amicon Ultra centrifugal unit with 3kDa, 10kDa and 30kDa molecular weight cut-off membranes (Millipore) were used to gauge the molecular mass of the antimicrobial.

7.3.9 *Bacteriocin purification*

Bacteriocins were purified by growing bacteriocin-producing cultures in TY broth at 30°C for 24 hours, after which cells were separated from the supernatant by centrifugation at 8000 rpm for 20 minutes. The cells were mixed with 250 ml of 70% isopropan-2-ol (IPA) 0.1% TFA and stirred for 3-4 hours at room temperature before centrifugation at 8000 rpm for 20 minutes after which point the cell pellet was discarded. The isopropan-2-ol was removed from the supernatant and was passed through a 5g 20 ml STRATA C18 SPE column pre-equilibrated with methanol and water. The column was washed with 20 ml 30% ethanol and bacteriocin activity was eluted with 20 ml 70% IPA 0.1% TFA. MALDI TOF Mass Spectrophotemetry was then performed. The sample was concentrated to 4 ml and applied to a semi prep Vydac C8 RP-HPLC column running a 25-60% acetonitrile 0.1% TFA gradient over 45 minutes at 2.5

ml/minute. Fractions were collected at 1 minute intervals and assayed for antimicrobial activity against the relevant indicator.

7.3.10 *Antimicrobial protein preparation*

The antimicrobial producer was grown in PDB at 25°C for 2 nights. Cell-free supernatant was generated by centrifugation at 8000 rpm for 20 minutes. Supernatant was passed through a Vivaflow 200 (Sartorius stedim biotech, Goettingen, Germany) with a 30 kDa cut-off membrane. Retentate was freeze-dried, and 140mg was resuspended in 20 ml 100 mM sodium acetate buffer at pH 5, and anion exchange chromatography was performed by passage through a DEAE Sepharose Hitrap column (GE Healthcare). The antimicrobial was eluted in 1M NaCl, and 5 samples were pooled together and desalted by passing through a millipore sirred cell unit fitted with a 5 kDa cutoff membrane (Millipore). 15ul of this sample was run on SDS-PAGE.

7.3.11 *Sodium dodecyl sulfate polyacrylamide gel electrophoresis*

SDS-PAGE of proteins was carried out using the Laemmlli protocol (Laemmli, 1970) and were stained with Coomassie brilliant blue R-250. A 2-212 marker (Roche) was used to determine the size of the protein.

7.4 RESULTS AND DISCUSSION

7.4.1 A high-throughput screen of strains isolated from fermented beverages reveals that antimicrobial producers are present at a low frequency

Since fermented foods have in the past been shown to be a good source of bacteriocin producers, it was anticipated that fermented beverages sourced from a number of different countries would serve as a good source of new antimicrobials. As the microbial composition of the fermentate and inoculation material of such beverages often differ (Marsh et al., 2013c), it was decided that there was merit in screening both the fermentate and inoculating material for bacteriocin producers.

Different agars were used to select for different components of the microbial populations, including GM17 for lactococci, MRS for lactobacilli, WL for fermentation kombucha isolates and TSA supplemented with yeast extract as a nutritious media that would facilitate the growth of many other strains. Approximately 63,000 colonies were picked and stocked in 384-well plates. The contents of six 384-well plates were stamped onto agar within Qtrays, which were then overlaid with different indicators to identify antimicrobial producers (**Figure 1**). The indicators selected were bacteriocin sensitive strains, i.e. *Lactococcus lactis* MG 1363 and *Lactobacillus bulgaricus* DPC 5838, representatives of pathogenic genera, i.e. *Listeria innocua* DPC 3572, *Salmonella typhimurium* LT2 and *Staphylococcus* aureus ATCC 25923, as well as a strain of *Leuconostoc mesenteroides* subsp. *cremoris* DPC 223 as a representative of a

species frequently found in kefir. Given that a number of producers of the lantibiotic lacticin 3147 have previously

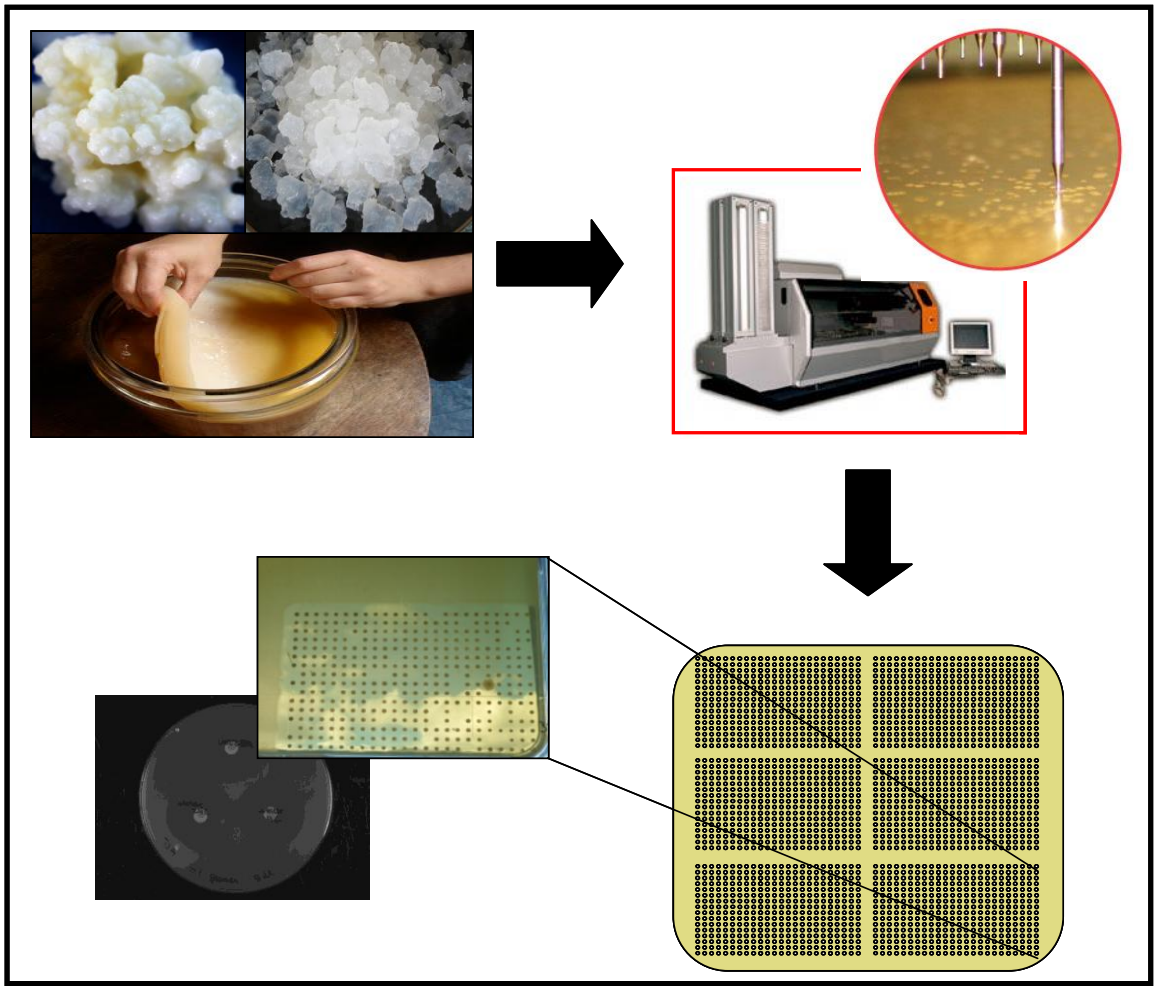

Figure 1

Flowchart showing the process for screening of the fermented beverages. Kefir, kombucha and water kefir were used as sources for the isolation of colonies, which were picked and stocked by the QPix2-XT. These were then stamped onto Qtrays, and overlaid with different indicators to assess for antimicrobial production.

been isolated from kefir (Ryan et al., 1996), *L. lactis* MGpMRC01, which is immune to lacticin 3147, was also used as an indicator to address the possibility that a considerable number of lacticin 3147 producers may be present in the samples.

Although these beverages and kefir, in particular, had in the past been good sources of novel bacteriocins such as lacticin 3147, ST8KF and others (Ryan et al., 1996, Aslim et al., 2005, Kojic et al., 2007), on this occasion relatively few antimicrobial producers were detected and only known lactococcins were identified from Irish kefir (see below). As water kefir has often been shown to have a similar microbial population to milk kefir, but had yet to be used as a source of antimicrobial producers, the fact that antimicrobial producers were not identified was disappointing. In the case of kombucha cultures, very few isolates grew on media other than WL media. Many of those that did grow were subsequently suspected to be yeast. This was despite the inclusion of the antifungal nystatin. While the lactic acid bacteria component of kombucha are known to be difficult to isolate, acetic acid bacteria are not commonly noted for their ability to produce bacteriocins (Qingping and Huaguang, 1999, Zhang et al., 2008), which may explain the lack of success in that regard.

It was found that acid production became an issue in that isolates cultured on MRS produced acid in such quantities that the growth of the indicator strains was severly retarded, making the identification of antimicrobial

producers extremely difficult. The addition of MOPS to buffer the agar did not solve the problem, and ultimately a representative number of MRS isolates from the frozen bank of 384-well plates were selected for direct well diffusion assays, but bacteriocin producers were not identified.

While the number of antimicrobial producers was lower than anticipated, a recent study involving bacteria revealed that only 0.08% of isolates produced bacteriocins with activity against *L. bulgaricus* and *Listeria innocua*, and that many of these had been previously characterised (Lakshminarayanan et al., 2013). It is thus clear that even highly diverse microbial communities can on occasion be of limited value with respect to the discovery of novel bacteriocins.

7.4.2 The lactococcin ABM complex was identified in several Irish kefir

A large number of colonies cultured from Irish kefir samples stamped on TSA-YE (from IR4 and IR6) or GM17 (from IR8) exhibited antimicrobial activity against *L. lactis* MGpMRC01. Representative colonies from each kefir sample were selected and the antimicrobial produced was further assayed via well-diffusion of the associated CFS. It was revealed that the antimicrobial was only active against other strains of *Lactococcus lactis* (i.e. the strains MG 1363, NZ 900, HP and DPC 3147). Sanger sequencing of 16S amplicons revealed that the three producers were of the species *Lactococcus lactis* and the peptide was therefore considered to be a lactococcin. Colony mass spectrometry revealed that the peptide mass profiles for three isolates were similar to one another, but none of the peaks identified corresponded to known lactococcins. The antimicrobial

from IR6, which was selected as a representative for further characterisation, was shown to be predominantly cell-associated. Initial investigations to determine how best to purify the antimicrobial established that the peptide was not soluble in ethanol or acetonitrile, was slightly soluble in methanol and isopropanol, was positively charged and active at low pH. Ultiamtely, the culture was grown in TY broth, and the cells were separated from the supernatant and washed in 70% IPA/0.1% TFA to release the peptide and purified by passing through a reverse phase SPE column. MALDI TOF mass spectrophotometry was performed on this sample and the presence of a 5774 Da peptide suggested the culture may be producing lactococcin A, which has a molecular mass of 5778 Da (**Figure 2**) (Holo et al., 1991). The sample was concentrated and run on HPLC, and the resulting fractions analysed. Peaks from the HPLC chromatogram corresponded to zones of inhibition in well diffusion assays, and mass spectrometry was performed on the resulting fractions. Peaks were identified which were found to closely relate to the masses of lactococcin A, lactococcin B and LcnN of lactococcin M respectively (van Belkum et al., 1991, van Belkum et al., 1992) (**Figure 2**).

These three bacteriocins are frequently co-produced by strains. The corresponding gene clusters have been identified on a single plasmid in *L. lactis* from an Irish cheese factory and starter culture previously (Morgan et al., 1995, Geis et al., 1983), and would appear to be adapted to fermented milk environments. From a kefir perspective, five *L. lactis* strains have previously been isolated from Serbian kefir that produce peptides that resemble lactococcins A and B (Kojic et al., 2007). These lactococcins, and producers

thereof, have shown potential as cheese ripening agents, whereby they accelerate flavour development by cell lysis

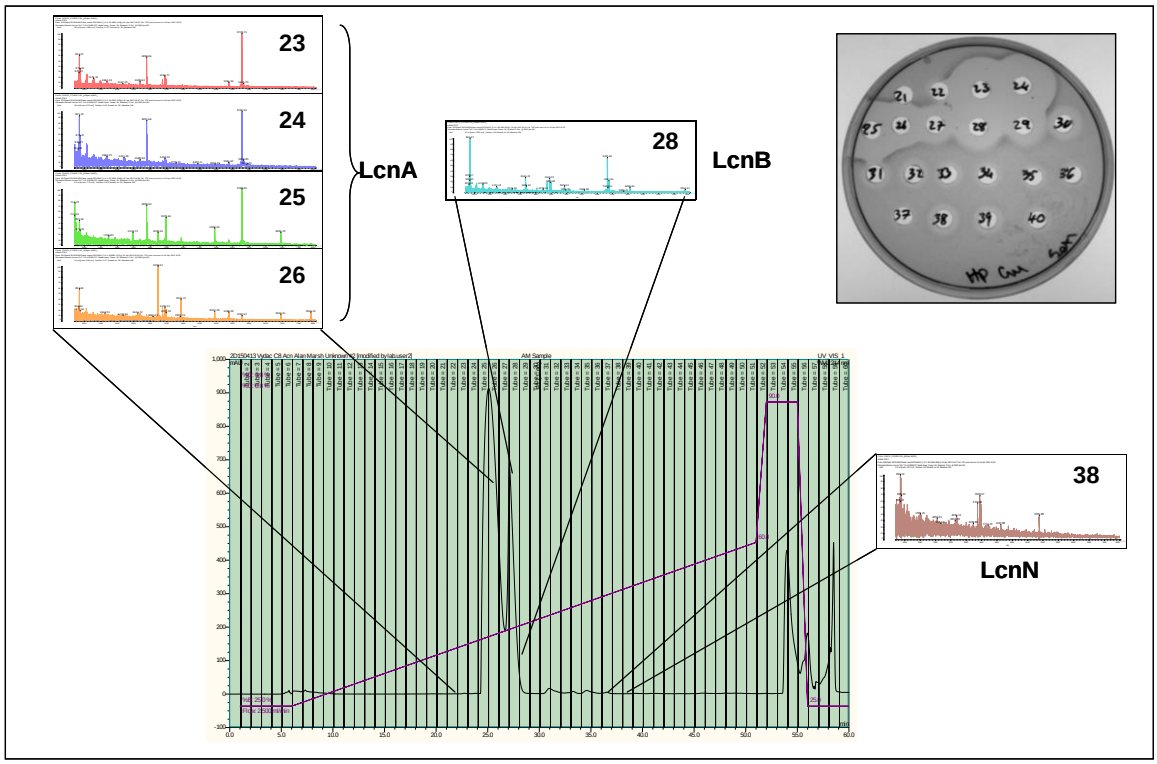

Figure 2

Chromatogram showing the different activities obtained from HPLC, and the masses of the different fractions were shown to correspond to Lactococcins A, B and LcnN of Lactococcin M. Fractions were also assayed against *L. lactis* HP to assess for antimicrobial activity (top right).

(Morgan et al., 1997, Morgan et al., 2002). Since these peptides have previously been associated with kefir isolates and are well-characterised, it was decided to instead focus on another, apparently novel, antimicrobial.

7.4.3 Identification of proteinaceous killer toxin produced by a kombucha-associated *Dekkera bruxellensis* strain

Colonies from UK and US-sourced kombucha, grown on WL agar supplemented with nystatin to inhibit fungal growth, were shown to inhibit *L. bulgaricus* DPC 5838. The antimicrobial was found to be sensitive to proteinase K indicating that the agent responsible for antimicrobial activity is proteinaceous in nature. 16S rRNA specific primers failed to generate amplicons when colony PCR was performed but internal transcribed specific (ITS) primers did generate a PCR product. Sanger sequencing of the ITS amplicon revealed that the producer was, in fact, the yeast *Dekkera bruxellensis*, that had grown despite the presence of nystatin, in the media, an antifungal which has been shown to inhbit this species (Rodrigues et al., 2001). This raised the question as to the nature of this antimicrobial. Yeast are capable of producing antimicrobials, known as mycocins or yeast killer toxins. These are analogous to bacteriocins in that they are generally active against closely related species and are proteinaceous by nature. However, they are larger than bacteriocins (usually 10-100 kDa), sometimes contain glycosyl residues and are generally, though not exclusively, active from pH 3-6 (Golubev, 2013). Despite reports of the antibacterial activity of some yeast killer toxins (Izgu and Altinbay, 1997, Meneghin et al., 2010, Fuentefria et al., 2008, Silva et al., 2011), there has yet to be a study that unambiguously

establishes that killer toxins inhibit prokaryotes. It is notable that a recent study has suggested that a killer toxin from *Pichia kudriavzevii* RY55 exhibits activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*, in addition to several species of *Aspergillus* (Bajaj et al., 2013), but further analysis of this antimicrobial is required.

To further investigate the nature of the antimicrobial that was the focus of our studies, the culture *D. bruxellensis* culture was centrifuged to generate cell free supernatant. When the CFS was filltered through molecular weight cutoff membranes, activity was found in the >30 kDa retentate, indicating the antimicrobial is a protein rather than a peptide. The protein was not active at pH2, and bound to anionic columns, revealing that it is negatively charged, both traits characteristic of mycocinogenic proteins. These characteristics meant that typical bacteriocin purification strategies could not be employed to purify the protein. The antimicrobial was shown to be heat stable after treatment at 60°C for 15mins, but activity was reduced following treatment for 15mins at 70°C and 80°C, with a loss of activity occurring when incubated at 90°C. This relative lack of heat stability is notable given the generally heat-labile nature of mycocins. Activity was retained through alkaline and acidic pHs (pH 3-8), which is also unusual for killer toxins. DEAE Sepharose anionic columns have been used to purify killer toxins in the past (Buzdar et al., 2011, Wang et al., 2007, Guo et al., 2013), and was therefore used to partially purify the antimicrobial. Faint bands of approximately 66 kDa, 55 kDa, 48 kDa, 34kDa and 31kDa could be seen when the partially purifed prep was run on SDS polyacrylamide gel, indicating the presence of several proteins in the preperation. The specific protein or proteins

responsible for antimicrobial activity remain unknown, and further purification is necessary. Additionally, the faint nature of the bands suggests there was a small yield from the purification process.

In addition to investigating the nature of the antimicrobial, its spectrum of activity was assessed in light of previous suggestions that such antimicrobials could be used to protect industrial fermentations from spoilage yeast (Ciani and Comitini, 2011, Santos et al., 2011). *Dekkera bruxellensis* is itself a species which has long been associated with the spoilage of wine (Woolfit et al., 2007). Since killer proteins are, like bacteriocins, typically active against closely related species, assays were carried out to screen species of *Dekkera* for sensitivity to the antimicrobial (**Table 3**). However, despite the use of several approaches including well diffusion, cell-cell antagonism and $NaCl^{+}$/smear assays, no inhibition of *Dekkera* was observed. Other genera of yeast, including those often identified in kombucha fermentations such as *Candida*, *Saccharomyces*, *Zygosaccharomyces* and *Pichia*, including the highly mycocin sensitive *Pichia heedi* NCYC 1490 strain (Vital et al., 2002), were also resistant. This resistance was apparent even when NaCl, which has been shown to enhance antimicrobial activity or enable activity (Golubev and Tomashevskaya, 2012, Llorente et al., 1997), was added. A recent study revealed a mycocin produced by *Kluyvermoyces lactis*, most active at alkaline pH, which was not active against closely related species, but rather “rare representatives” of yeast genera (Golubev, 2013), suggesting that further assays with a larger range of indicator taxa should be considered in the future. A number of bacteria were also used as

indicators, but activity was only reliably and consistently shown against different strains of *Lactobacillus bulgaricus* (**Table 2**).

The *Lactobacillus* genus is found, albeit at low abundance in kombucha fermentations, so activity against this bacterium might provide a competitive advantage to *D. bruxellensis* in this environment. The fact that this antimicrobial is being produced by two different kombuchas (sourced from the UK and US) establishes that its occurrence is not unique.

While most known mycocins are active at acidic pHs and are heat labile, recent studies are providing information about proteins that do not conform with these general rules and which are active against non-related species (Golubev, 2013, Buzzini et al., 2004, Ochigava et al., 2011, Antunes and Aguiar, 2012), and thus the *D. bruxellensis*-associated protein may also be an atypical mycocin. While the killer phenotype has been associated with *D. bruxellensis* on one previous occasion, with activity against *Candida* and *Zygosaccharomyces* (Antunes and Aguiar, 2012), a mycocin has yet to be purified from this genus.

However, the antimicrobial that was the emerged from our study does not show activity against any of the yeasts screened, and further characterisation is required to confirm that it is mycocinogenic in nature. Regardless, further screening of the yeast component of these and other fermented foods for producers of antimicrobials merits consideration.

Table 2

A list of the *Lactobacillus bulgaricus* strains (A), *Lactobacillus* strains (B) and other strains (C) used as bacterial indicators in screening for antibacterial activity

A) Sensitive *Lactobacillus bulgaricus* strains	**Zone**
Lactobacillus bulgaricus DPC 5396	1mm
Lactobacillus bulgaricus DPC 1184	2mm
Lactobacillus bulgaricus DPC 6104	3mm
Lactobacillus bulgaricus DPC 5384	3mm
Lactobacillus bulgaricus DPC 3966	2mm
Lactobacillus bulgaricus DPC 1185	1mm
Lactobacillus bulgaricus DPC 1187	1mm
Lactobacillus bulgaricus DPC 1181	1mm
Lactobacillus bulgaricus DPC 1183	1.5mm
Lactobacillus bulgaricus DPC 3322	3mm
Lactobacillus bulgaricus DPC 5838	3mm

B) Other *Lactobacillus* spp.	**Zone**
Lactobacillus amylovorus DPC 6499	X
Lactobacillus bulgaricus DPC 5406	X
Lactobacillus bulgaricus DPC 1186	X
Lactobacillus bulgaricus DPC 1182	X
Lactobacillus casei DPC 6063	X
Lactobacillus crispatus LMG 9479	X
Lactobacillus fermentum LMG 6902	X
Lactobacillus gallinarum LMG 9435	X
Lactobacillus mucosae DPC 6426	X
Lactobacillus murinus DPC 6139	X
Lactobacillus paracheseii NFBC 338	X
Lactobacillus pentosus DPC 6140	X
Lactobacillus plantarum DPC 4850	X
Lactobacillus rhamnosus LMG 6400	X
Lactobacillus ruminis DPC 6419	X
Lactobacillus salivarius DPC 6196	X
Lactobacillus salivarius DPC 6488	X
Lactobacillus salivarius UCC 118	X

C) Other Indicators	Zone
Bacillus cereus ATCC 9139	X
Bacillus subtilis LMG 8198	X
Enterococcus faecalis E265	X
Lactococcus lactis HP	X
Lactococcus lactis MG 1363	X
Lactococcus lactis NZ 900	X
Lactococcus lactis DPC 3147	X
Leuconostoc cremoris DPC 223	X
Listeria innocua DPC 3572	X
Pediococcus pentosaceus LMG11488	X
Salmonella typhimurium LT2	X
Staphylococcus aureus ATCC 25923	X
Streptococcus faecalis DPC 3556	X
Streptococcus faecalis DPC 3557	X
Streptococcus agalactiae LMG 14694/ATCC 13813	X
Streptococcus uberis DPC 5344	X
Streptococcus mutans NCTC 10449	X

Table 3

A list of the yeast indicators used in the screening for antifungal activity from the *Dekkera bruxellensis* antimicrobial protein

Strain	Zone
Candida intermedia DPC 6270	X
Candida intermedia DPC 6271	X
Zygosaccyaromyces visicolier DPC 5734	X
Debaryomyces hansenii DPC 5729	X
Debaryomyces Kloackeri var. *major WSH 50 DPC 5731*	X
Candida Catenulata WSYC 319 DPC 5730	X
Yarrowia lipolytica DPC 6266	X
Debaryomyces hansenii DPC 6264	X
Debaryomyces hansenii DPC 6265	X
Dekkera anomala DSM 70727	X
Dekkera bruxellensis DSM 70001	X
Dekkera custersiana DSM 70736	X
Dekkera naardenensis DSM 70743	X
Zygosaccharomyces bisporus DSM 70415	X
Pichia anomala DSM 6766	X
Pichia heedii NCYC 1490	X
Rhodotorula mucilaginosa (unspecified strain)	X
Candida parapsilosis(unspecified strain)	X
Saccharomyces cerevisiae (unspecified strain)	X

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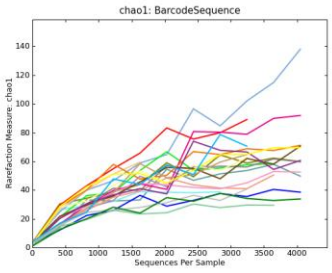
APPENDIX

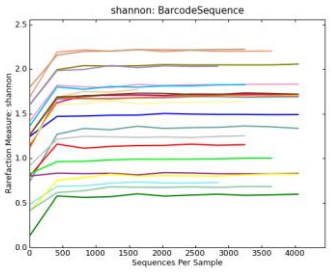
Appendix A: Supplemental information for Chapter 4

Sequencing-based analysis of the bacterial and fungal composition of kefir grains and milks from multiple sources

A) Grain

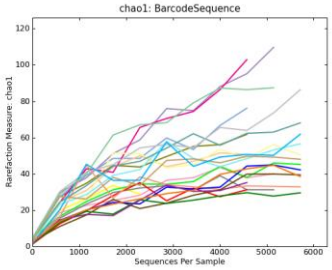


Chao1

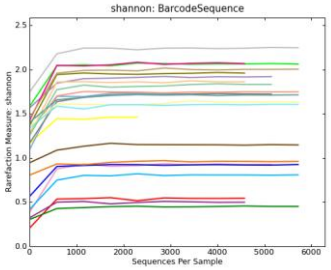


Shannon

B) Milk

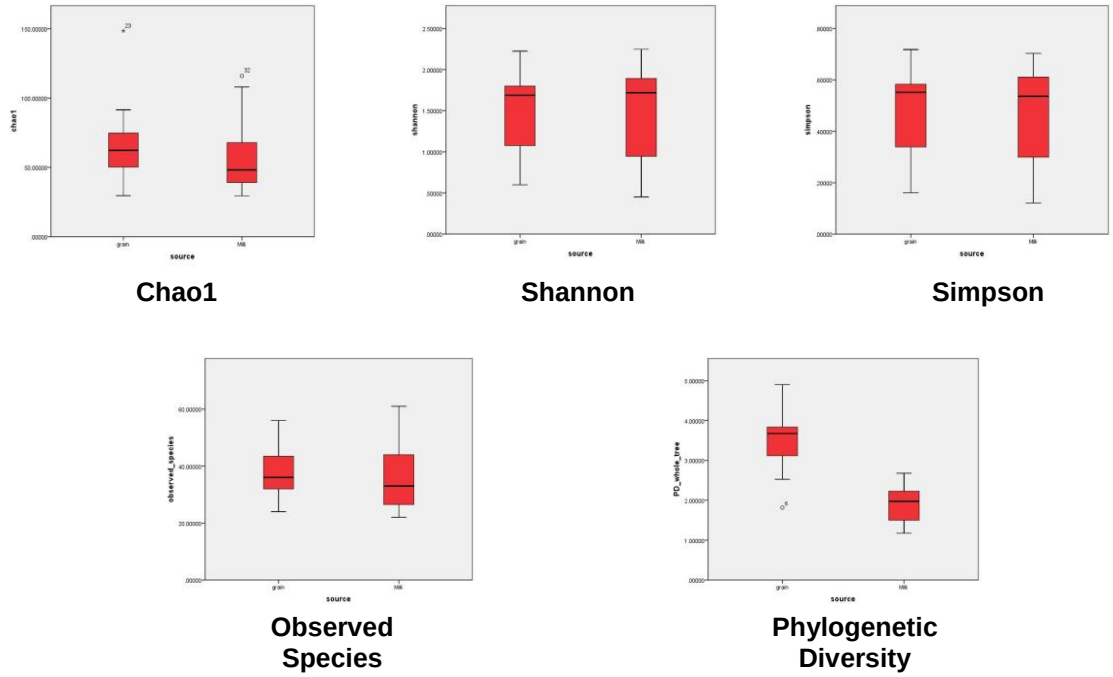


Chao1

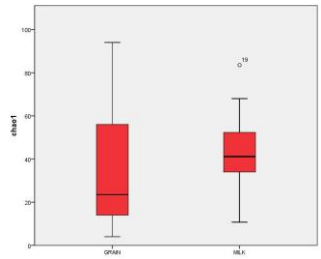


Shannon

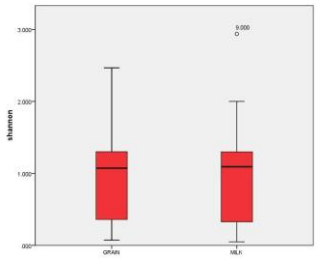
Supplemental Figure 1: Rarefactions for the 16S kefir milk and grain Chao1 and Shannon indices where A = Grains and B = Milks



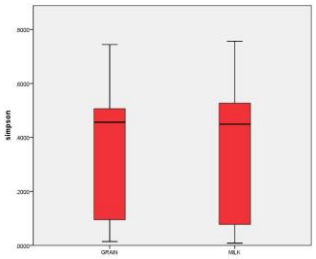
Supplemental Figure 2: Box plots of the 16S alpha diversity



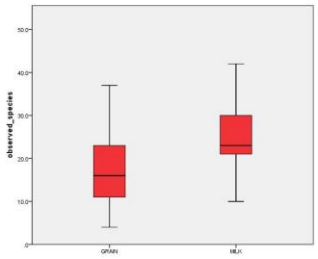
Chao1



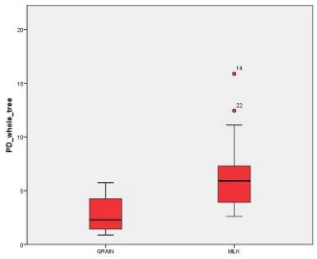
Shannon



Simpson



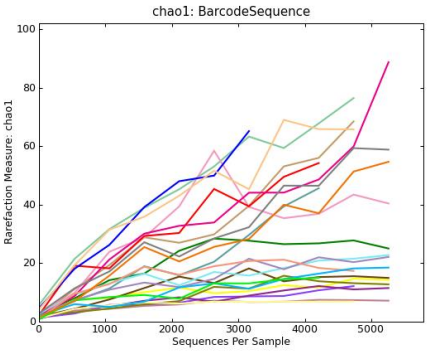
Observed Species



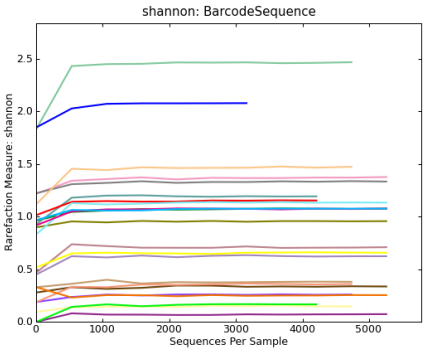
Phylogenetic Diversity

Supplemental Figure 3: Box plots of the ITS alpha diversity

A) Grain

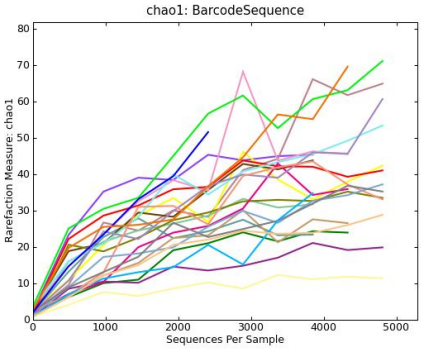


Chao1

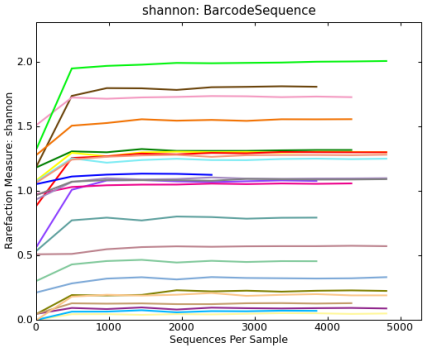


Shannon

B) Milk



Chao1



Shannon

Supplemental Figure 4: Rarefactions of the ITS kefir milk and grain Chao1 and Shannon indices where A = Grains and B = Milks

Kefir Name	Origin	Source		Additional Info
US1	United States	Company	Cultures For Health	Dehydrated
US3	United States	Company	Your Kefir Source	
Ger1	Germany	Company	Vital Drinks	
UK1	United Kingdom	Company	Kefir Shop	
US2	United States	Company	Kefir Lady	
US5	United States	Company	Rejoice in Life	Dehydrated
US4	United States	Company	Savvy Teas and Herbs	Unpasteurised Milk
IT1	Italy	Individual Contact		
FR1	France	Individual Contact		
Ger2	Germany	Individual Contact		
SP1	Spain	Individual Contact		
Bel1	Belgium	Individual Contact		
UK2	United Kingdom	Individual Contact		
UK3	United Kingdom	Individual Contact		
UK4	United Kingdom	Individual Contact		
CA1	Canada	Individual Contact		
UK5	United Kingdom	Individual Contact		

Supplemental Table 1: Sources of kefir samples

Grain	Chao1	Simpson	Shannon	Phylogenetic Diversity	Observed Species
BEL1	89.857143	0.322229747	1.151474964	4.90177	47
CA1	60.428571	0.356148569	1.000360006	3.61941	36
FR1	62.428571	0.24393226	0.831990501	3.45632	38
GER1	41.428571	0.208659552	0.733845708	3.73523	32
GER2	59.428571	0.576212719	1.778737293	3.02014	35
IR1	29.6	0.161181917	0.679678613	1.81705	24
IR10	58.363636	0.687076709	2.055196176	3.74655	46
IR2	62.272727	0.195994136	0.821974127	3.9069	45
IR3	66	0.682011448	2.033049794	2.52558	31
IR4	63.2	0.515064403	1.631761195	3.67431	36
IR5	41	0.357235569	1.246135312	2.70234	26
IR6	91.5	0.583847951	1.719909851	3.88363	39
IR8	77.333333	0.575493025	1.82049819	3.57961	32
IR9	72.25	0.718006417	2.223508843	3.62395	34
IT1	55.5	0.66535649	1.832230731	3.21449	38
SP1	39.428571	0.581939422	1.494818705	2.64333	30
UK1	63.375	0.429743322	1.358610597	4.35213	42
UK2	84.111111	0.51106434	1.710162536	4.64035	56
UK3	39.166667	0.174024314	0.599510645	3.79165	30
US1	60	0.551344418	1.71823702	4.35363	41
US2	90.428571	0.579748985	1.728058155	3.70098	51
US3	45	0.705011851	2.203295841	2.84788	32
US5	148.5	0.575570837	1.689024412	3.6874	47

Milk	Chao1	Simpson	Shannon	Phylogenetic Diversity	Observed Species
BEL1	47.5	0.374153184	1.152567837	1.2791	25
CA1	46.75	0.702884236	2.06730088	2.17078	33
FR1	30	0.121015053	0.451194038	1.56664	23
GER1	29.5	0.552708593	1.461280595	1.38427	25
GER2	57	0.623888494	1.862857158	1.83503	33
IR1	87.428571	0.539376805	1.832544164	2.13412	48
IR10	60	0.643189906	1.960675459	2.21297	43
IR2	38	0.133633116	0.500955457	1.64722	23
IR3	116	0.591569875	1.922007927	2.67777	51
IR4	51.666667	0.535551288	1.633770127	2.00318	40
IR5	108	0.663702199	2.247030145	2.31915	48
IR6	102.75	0.670655411	2.074999006	2.11627	45
IR8	65.2	0.21038868	0.80972062	2.25856	38
IR9	48.142857	0.678040313	2.003182555	2.24123	37
IT1	60	0.589212289	1.606192441	1.9759	36
SP1	31	0.153816039	0.543628859	1.4283	22
UK1	40.25	0.271931852	0.92880951	1.85377	29
UK2	41	0.264076933	0.921660013	1.46781	26
UK3	43.5	0.326426064	0.963303442	1.1724	27
US1	31.666667	0.524193804	1.723050492	1.47926	27
US2	86.666667	0.52825382	1.717945614	2.63327	61
US3	32.8	0.598057595	1.748797963	1.52136	30
US5	70.5	0.524047565	1.72577908	2.48339	45

Supplemental Table 2: 16S alpha diversities

Grain	Chao1	Simpson	Shannon	Phylogenetic Diveristy	Observed Species
BEL1	14.66666667	0.102980374	0.334582493	0.8708	13
CA1	4	0.551584484	1.302291763	1.03134	4
FR1	25.14285714	0.484204239	1.073251729	3.74179	20
GER1	15	0.259954001	0.657617505	1.58687	12
GER2	26.5	0.198410653	0.622425	1.74371	16
IR1	7	0.031239654	0.147844986	1.53319	7
IR10	7.333333333	0.277525423	0.706306939	1.20657	7
IR2	11	0.013961747	0.071403438	1.38798	9
IR4	94	0.494759438	1.07495927	5.74017	26
IR5	18.5	0.506409666	1.075015608	2.28067	11
IR6	68.5	0.095145764	0.37856945	4.25407	23
IR8	14	0.456511803	0.955377948	1.19739	9
IR9	11	0.077047309	0.257707014	1.34051	8
IT1	23.5	0.446011764	1.132317594	2.5282	16
SP1	56	0.464657173	1.151908449	5.00048	23
UK1	41.375	0.54433288	1.372674234	3.11514	30
UK2	75.33333333	0.696429187	2.081158637	4.66894	30
UK3	53	0.055138315	0.254210336	3.08227	20
UK4	62	0.545478875	1.332518118	5.232	23
UK5	17.2	0.090414508	0.359503994	1.67322	16
US1	14	0.035466069	0.166738874	1.94581	11
US2	50	0.462742166	1.191925426	1.42621	14
US3	72	0.521756462	1.473312785	5.15106	34
US5	82.33333333	0.744162084	2.467399236	4.28862	37

Milk	Chao1	Simpson	Shannon	Phylogenetic Diveristy	Observed Species
BEL1	47.33333333	0.619656262	1.809163769	4.91291	29
CA1	50.5	0.07776884	0.327360687	6.63581	23
FR1	24.33333333	0.527069894	1.316372942	2.84088	15
GER1	44.5	0.525933215	1.300491676	3.985	25
GER2	61	0.504229437	1.098739283	4.97076	26
IR1	10.75	0.008172839	0.049624645	2.59532	10
IR10	68	0.181660438	0.572385267	8.19713	28
IR2	23	0.015763689	0.092257005	5.24963	16
IR4	34.2	0.488282384	1.057285126	6.66776	21
IR5	41	0.011386143	0.069382641	3.91416	13
IR6	24.2	0.024167974	0.129321558	4.41839	17
IR8	34	0.03857195	0.225372863	11.10525	30
IR9	46	0.268675267	1.076586742	15.87415	37
IT1	54	0.449213635	1.249934207	3.7605	30
SP1	41.11111111	0.495165149	1.299695516	10.41399	35
UK1	47.5	0.635171659	1.732553533	6.89271	30
UK2	52.33333333	0.491523022	1.132283402	3.45618	22
UK3	83.5	0.567377268	1.555601362	7.29881	31
UK4	34.75	0.430419871	1.093167883	3.38308	21
UK5	35	0.544022869	1.280250436	6.24733	22
US1	64.66666667	0.571574795	2.000269036	12.44054	42
US2	23.625	0.240205749	0.79374936	3.38219	21
US3	55	0.039897894	0.195217424	5.91388	22
US5	33	0.102658629	0.453759347	6.36783	24

Supplemental Table 3: ITS alpha diversities

	BEL1	CA1	FR1	GER1	GER2	IR1	IR2	IR3	IR4	IR5	IR6	IR8
Phylum												
Proteobacteria	0.004454	0	0.011704	0.003378	0.199126	0.01558	0.020174	0.033619	0.270081	0.032357	0.691367	0.266499
Firmicutes	0.994432	0.997711	0.987913	0.992629	0.798932	0.98416	0.978735	0.965392	0.72796	0.96569	0.30771	0.732383
Bacteroidetes	0	0	0	0.003071	0	0	0.000909	0	0.001679	0	0	0
Actinobacteria	0	0	0	0	0	0	0	0	0	0	0	0
Other	0.001114	0.002289	0.000384	0.000921	0.001943	0.00026	0.000182	0.000989	0.00028	0.001953	0.000923	0.001119
Family												
Acetobacteraceae	0.003341	0	0.011512	0.002764	0.199126	0.01558	0.019993	0.033619	0.269801	0.032357	0.690905	0.266499
Lachnospiraceae	0.003619	0	0.002302	0.002457	0	0.002597	0.001272	0	0.002519	0	0.00277	0.001957
Ruminococcaceae	0.082127	0	0	0	0.002428	0.018437	0.006361	0	0.013434	0	0	0
Lactobacillaceae	0.895601	0.989827	0.978319	0.981572	0.775134	0.955077	0.948201	0.960448	0.706409	0.947559	0.2994	0.723154
Streptococcaceae	0.005568	0	0.004797	0	0.01797	0.006232	0.021628	0.002966	0.002519	0.014784	0.004155	0.005034
Leuconostocaceae	0.003062	0	0.001343	0	0	0	0	0	0	0	0	0
Clostridiaceae	0	0	0	0.003686	0	0	0	0	0	0	0	0
Enterococcaceae	0	0	0	0	0	0	0	0	0	0	0	0
Propionibacterineae	0	0	0	0	0	0	0	0	0	0	0	0
Bifidobacteriaceae	0	0	0	0	0	0	0	0	0	0	0	0
Bacteroidaceae	0	0	0	0	0	0	0	0	0	0	0	0
Rikenellaceae	0	0	0	0	0	0	0	0	0	0	0	0
Other	0.006682	0.010173	0.001727	0.009521	0.005342	0.002077	0.002545	0.002966	0.005318	0.0053	0.00277	0.003356
Genus												
Acetobacter	0.003341	0	0.011512	0.002764	0.199126	0.01558	0.019993	0.033619	0.269801	0.032357	0.690905	0.266499
Faecalibacterium	0.0799	0	0	0	0	0.018177	0.005634	0	0.012594	0	0	0
Ruminococcaceae Incertae Sedis	0.001392	0	0	0	0	0	0	0	0	0	0	0
Lactobacillus	0.895601	0.989827	0.978319	0.981572	0.775134	0.955077	0.948201	0.960448	0.706409	0.947559	0.2994	0.723154
Lactococcus	0.005568	0	0.004797	0	0.01797	0.006232	0.021628	0.002966	0.002519	0.014784	0.004155	0.005034
Leuconostoc	0.003062	0	0.001343	0	0	0	0	0	0	0	0	0
Allobaculum	0	0.005595	0	0	0	0	0	0	0.003079	0.001395	0	0
Rickenella	0	0	0	0.001536	0	0	0	0	0	0	0	0
Clostridium	0	0	0	0.003378	0	0	0	0	0	0	0	0
Enterococcus	0	0	0	0	0	0	0	0	0	0	0	0
Bifidobacterium	0	0	0	0	0	0	0	0	0	0	0	0
Bacteroides	0	0	0	0	0	0	0	0	0	0	0	0
Alistipes	0	0	0	0	0	0	0	0	0	0	0	0
Other	0.011136	0.004578	0.004029	0.010749	0.007771	0.004934	0.004544	0.002966	0.005598	0.003905	0.00554	0.005313

Supplemental Table 4: Relative abundances for the 16S grain

	BEL1	CA1	FR1	GER1	GER2	IR1	IR2	IR3	IR4	IR5	IR6	IR8
Phylum												
Proteobacteria	0.011737	0.430899	0	0.00261	0.102909	0.7524	0.008217	0.256513	0.016942	0.605441	0.293759	0.904079
Fimicutes	0.988263	0.56881	1	0.99739	0.897091	0.247419	0.991592	0.74314	0.98291	0.393271	0.705631	0.09577
Actinobacteria	0	0	0	0	0	0	0	0	0	0	0	0
Other	0	0.000291	0	0	0	0.000181	0.000191	0.000347	0.000149	0.001288	0.00061	0.000151
Family												
Acetobacteraceae	0.011737	0.430899	0	0.00261	0.102909	0.7524	0.008217	0.256513	0.016942	0.605441	0.293759	0.904079
Lactobacillaceae	0.121909	0.20934	0.004622	0.425802	0.25657	0.071545	0.004204	0.605071	0.223362	0.23519	0.199024	0.064653
Streptococcaceae	0.784194	0.330957	0.942142	0.540268	0.559077	0.163195	0.930633	0.101598	0.6524	0.148583	0.489124	0.028701
Leuconostocaceae	0.080751	0.028077	0.052757	0.024609	0.079238	0.012317	0.056373	0.03265	0.104325	0.007405	0.015044	0.001511
Propionibacterinaceae	0	0	0	0	0	0	0	0	0	0	0	0
Other	0.001408	0.000727	0.000478	0.006711	0.002207	0.000543	0.000573	0.004168	0.002972	0.003381	0.003049	0.001057
Genus												
Acetobacter	0.011737	0.430899	0	0.00261	0.102909	0.752038	0.008217	0.256339	0.016942	0.604958	0.293759	0.903776
Lactobacillus	0.121909	0.20934	0.004622	0.425802	0.25657	0.071545	0.004204	0.605071	0.223362	0.235029	0.199024	0.064653
Lactococcus	0.784038	0.330957	0.942142	0.540268	0.559077	0.163195	0.930633	0.101598	0.652251	0.148261	0.489124	0.028701
Leuconostoc	0.080751	0.028077	0.052757	0.024609	0.079238	0.012317	0.056373	0.03265	0.104325	0.007405	0.015044	0.001511
Propionibacterium	0	0	0	0	0	0	0	0	0	0	0	0
Other	0.001565	0.000727	0.000478	0.006711	0.002207	0.000906	0.000573	0.004342	0.003121	0.004346	0.003049	0.00136

Supplemental Table 5: Relative abundances for the 16S milk

	BEL1	FR1	GER1	GER2	IR1	IR2	IR4	IR5	IR6	IR8	IR9	IR10
Phylum												
Ascomycota	1	0.99945	1	0.999846	1	1	0.99712	0.999623	0.999181	0.999465	0.999797	0.999636
Other	0	0.00055	0	0.000154	0	0	0.00288	0.000377	0.000819	0.000535	0.000203	0.000364
Family												
Saccharomycetaceae	1	0.99945	0.999011	0.999846	1	1	0.99712	0.999623	0.998567	0.999465	0.999797	0.999636
Davidiellaceae	0	0	0	0	0	0	0	0	0	0	0	0
Trichocomaceae	0	0	0	0	0	0	0	0	0	0	0	0
Pichiaceae	0	0	0	0	0	0	0	0	0	0	0	0
Dothioraceae	0	0	0	0	0	0	0	0	0	0	0	0
Other	0	0.00055	0.000989	0.000154	0	0	0.00288	0.000377	0.001433	0.000535	0.000203	0.000364
Genus												
Kazachstania	0.0024	0.006047	0	0.080488	0.987949	0.994039	0.420266	0.532264	0.952498	0.650864	0.038774	0.835333
Kluyveromyces	0.051514	0.37768	0.148179	0	0.004544	0	0	0.00717	0.005938	0	0	0.009269
Naumovozyma	0	0	0	0.024564	0.002371	0	0.575594	0.459245	0.003481	0.348245	0.960211	0.155035
Saccharomyces	0	0	0	0	0	0	0	0.000943	0.002867	0	0	0
Davidiella	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium	0	0	0	0	0	0	0	0	0	0	0	0
Pichia	0	0	0	0	0	0	0	0	0	0	0	0
Dekkera	0	0	0	0	0	0	0	0	0	0	0	0
Aureobasidium	0	0	0	0	0	0	0	0	0	0	0	0
Other	0.946086	0.616273	0.851821	0.894948	0.005136	0.005961	0.00414	0.000377	0.035217	0.000891	0.001015	0.000364
Species												
Kazachstania barnetti	0.0024	0.006047	0	0	0	0	0	0	0	0	0	0
Kluyveromyces marxianus	0.051514	0.37768	0.148179	0	0.004544	0	0	0.00717	0.005938	0	0	0.009269
Kazachstania unispora	0	0	0	0.080488	0.987949	0.994039	0.420266	0.532264	0.952498	0.650864	0.038774	0.835333
Naumovozyma castelli	0	0	0	0.024564	0.002371	0	0.575594	0.459245	0.003481	0.348245	0.960211	0.155035
Saccharomyces cerevisiae	0	0	0	0	0	0	0	0.000943	0	0	0	0
Davidiella tassiana	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium sp. *Vega 347*	0	0	0	0	0	0	0	0	0	0	0	0
Pichia kudriavzevii	0	0	0	0	0	0	0	0	0	0	0	0
Dekkera anomala	0	0	0	0	0	0	0	0	0	0	0	0
Other	0.946086	0.616273	0.851821	0.894948	0.005136	0.005961	0.00414	0.000377	0.038084	0.000891	0.001015	0.000364

Supplemental Table 6: Relative abundances for the ITS grain (Part 1)

	IT1	SP1	UK1	UK2	UK3	UK4	UK5	US1	US2	US3	US5
Phylum											
Ascomycota	0.999812	0.998465	0.99963	0.998526	1	0.999309	0.999805	0.999086	1	0.997866	0.999453
Other	0.000188	0.001535	0.00037	0.001474	0	0.000691	0.000195	0.000914	0	0.002134	0.000547
Family											
Saccharomycetaceae	0.999812	0.98531	0.99963	0.998232	0.998414	0.99741	0.999805	0.999086	1	0.997672	0.999179
Davidiellaceae	0	0.002193	0	0	0	0	0	0	0	0	0
Trichocomaceae	0	0.001316	0	0	0	0	0	0	0	0	0
Pichiaceae	0	0.005701	0	0	0	0	0	0	0	0	0
Dothioraceae	0	0	0	0	0.000881	0	0	0	0	0	0
Other	0.000188	0.005481	0.00037	0.001768	0.000705	0.00259	0.000195	0.000914	0	0.002328	0.000821
Genus											
Kazachstania	0.045198	0.663451	0.046615	0.277925	0.003876	0.42058	0.029854	0.992455	0.696672	0.655801	0.57049
Kluyveromyces	0.249906	0.003947	0.384573	0.056882	0.014623	0.040573	0.009171	0	0.085102	0.139697	0.156584
Naumovozyma	0	0.305196	0.004624	0.183319	0.005814	0	0	0.001829	0.00134	0.015716	0.010129
Saccharomyces	0	0	0	0	0	0	0	0	0	0.004463	0
Davidiella	0	0.002193	0	0	0	0	0	0	0	0	0
Penicillium	0	0.001316	0	0	0	0	0	0	0	0	0
Pichia	0	0.005701	0	0	0	0	0	0	0	0	0
Dekkera	0	0.01184	0	0	0	0	0	0	0	0	0
Aureobasidium	0	0	0	0	0.000881	0	0	0	0	0	0
Other	0.704896	0.006358	0.564188	0.481874	0.974806	0.538847	0.960976	0.005716	0.216886	0.184323	0.262798
Species											
Kazachstania barnetti	0	0	0	0	0	0	0	0	0	0	0
Kluyveromyces marxianus	0.249906	0.003947	0.384573	0.056882	0.014623	0.040573	0.009171	0	0.085102	0.139697	0.156584
Kazachstania unispora	0.045198	0.663451	0.046615	0.277041	0.003876	0.42058	0.029854	0.992455	0.696002	0.655801	0.57049
Naumovozyma castelli	0	0.305196	0.004624	0.183319	0.005814	0	0	0.001829	0.00134	0.015716	0.010129
Saccharomyces cerevisiae	0	0	0	0	0	0	0	0	0	0	0
Davidiella tassiana	0	0.002193	0	0	0	0	0	0	0	0	0
Penicillium sp. *Vega 347*	0	0.001316	0	0	0	0	0	0	0	0	0
Pichia kudriavzevii	0	0.005701	0	0	0	0	0	0	0	0	0
Dekkera anomala	0	0.01184	0	0	0	0	0	0	0	0	0
Other	0.704896	0.006358	0.564188	0.482759	0.975687	0.538847	0.960976	0.005716	0.217556	0.188785	0.262798

Supplemental Table 6: Relative abundances for the ITS grain (Part 2)

	BEL1	FR1	GER1	GER2	IR1	IR2	IR4	IR5	IR6	IR8	IR9	IR10	IT1	SP1	UK1	UK2	UK3	UK4	UK5	US1	US2	US3	US5
Phylum																							
Ascomycota	0.995031	0.999554	1	0.998212	0.952918	0.995828	0.998547	0.998515	0.998054	0.990595	0.938609	0.893837	1	0.990837	0.992322	0.999631	0.996476	0.999259	0.998893	0.89835	0.999275	0.999717	0.995446
Basidiomycota	0	0	0	0.000993	0.003879	0.000927	0	0	0.001297	0	0.010791	0.008748	0	0.003869	0.00664	0	0	0	0	0.015449	0	0	0
Uncultured Fungus	0.004472	0	0	0	0.003527	0.0017	0	0	0	0.001447	0.008633	0.007952	0	0.001425	0	0	0	0	0	0.013343	0	0	0.002024
Other	0.000497	0.000446	0	0.000795	0.039676	0.001545	0.001453	0.001485	0.000649	0.007958	0.041966	0.089463	0	0.003869	0.001038	0.000369	0.003524	0.000741	0.001107	0.072858	0.000725	0.000283	0.00253
Family																							
Saccharomycetaceae	0.994783	0.997992	0.999211	0.997815	0.949921	0.994747	0.997717	0.998515	0.997622	0.985893	0.907914	0.887078	0.999446	0.64325	0.988172	0.998894	0.990257	0.999074	0.998339	0.833743	0.999275	0.997449	0.992158
Davidiellaceae	0	0	0	0	0	0	0	0	0	0	0.008393	0	0	0	0	0	0	0	0	0	0	0	0
Trichocomaceae	0	0	0	0	0.000705	0	0	0	0	0	0.004556	0.00159	0	0	0	0	0	0	0	0	0	0	0
Pichiaceae	0	0	0	0	0	0.000927	0	0	0	0	0	0	0	0.3427	0.00332	0	0.004768	0	0	0	0	0.001276	0.003289
Tremellomycetes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Wallemiomycetes	0	0	0	0	0.002557	0	0	0	0	0	0	0.005765	0	0	0	0	0	0	0	0	0	0	0
Herpotrichiellaceae	0	0	0	0	0	0	0	0	0	0.001085	0	0	0	0	0	0	0	0	0	0	0	0	0
Teratosphaeriaceae	0	0	0	0	0	0	0	0	0	0	0.001199	0	0	0	0	0	0	0	0	0.001053	0	0	0
Valsaceae	0	0	0	0	0	0	0	0	0	0	0.008393	0	0	0	0	0	0	0	0	0	0	0	0
Debaryomycetaceae	0	0	0	0	0	0	0	0	0	0	0.003357	0	0	0	0	0	0	0	0	0	0	0	0
Phaffomycetaceae	0	0	0	0	0	0	0	0	0	0	0.002878	0	0	0	0	0	0	0	0	0	0	0	0
Malasseziaceae	0	0	0	0	0	0	0	0	0	0	0.002158	0	0	0	0	0	0	0	0	0	0	0	0
Bondarzewiaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00664	0	0	0	0	0	0	0	0
Dermataceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.006496	0	0	0
Pezizaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.003511	0	0	0
Ganodermataceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.005794	0	0	0
Tricholomataceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.005267	0	0	0
Other	0.005217	0.002008	0.000789	0.002185	0.046817	0.004326	0.002283	0.001485	0.002378	0.013022	0.061151	0.105567	0.000554	0.01405	0.001868	0.001106	0.004975	0.000926	0.001661	0.144136	0.000725	0.001276	0.004554
Genus																							
Kazachstania	0.012174	0.346497	0.056816	0.548272	0.948598	0.991965	0.399045	0.997277	0.988973	0.982094	0.068585	0.885288	0.157029	0.627774	0.467939	0.004425	0.360904	0.716296	0.519195	0.600597	0.869471	0.980726	0.008854
Kluyveromyces	0.501615	0.033244	0.338726	0	0.000529	0	0	0	0	0.000904	0.001679	0.001193	0.114724	0.001018	0.359203	0.379425	0.058665	0.039815	0.046881	0.044066	0.05753	0.010062	0.024285
Naumovozyma	0	0.593039	0	0	0	0	0.594107	0	0.005622	0.000904	0.818705	0	0	0.009978	0.017639	0	0.5483	0	0	0.018083	0	0	0.005818
Saccharomyces	0	0	0	0	0	0	0	0	0	0	0.009353	0	0	0	0	0	0.001036	0	0	0	0	0.000709	0
Davidiella	0	0	0	0	0	0	0	0	0	0	0.008393	0	0	0	0	0	0	0	0	0	0	0	0
Pichia	0	0	0	0	0	0.000927	0	0	0	0	0	0	0	0.3427	0.00332	0	0.004768	0	0	0	0	0.001276	0.003289
Dekkera	0.002733	0.001116	0	0	0	0	0.00332	0	0	0	0	0	0.003695	0.002647	0.00498	0.002581	0.00228	0	0	0.001229	0	0.004677	0
Zygosaccharomyces	0	0	0	0	0.000441	0	0	0	0.002378	0.001266	0	0	0	0	0	0	0	0	0	0.001053	0	0.000709	0
Wallemia	0	0	0	0	0.002557	0	0	0	0	0	0	0.005765	0	0	0	0	0	0	0	0	0	0	0
Eurotium	0	0	0	0	0.000705	0	0	0	0	0	0	0.00159	0	0	0	0	0	0	0	0	0	0	0
Microdochium	0	0	0	0	0	0	0	0	0	0.001809	0	0	0	0	0	0	0	0	0	0	0	0	0
Cryptococcus	0	0	0	0	0	0	0	0	0	0.000904	0.006235	0	0	0	0	0	0	0	0	0	0	0	0
Teratosphaeria	0	0	0	0	0	0	0	0	0	0	0.001199	0	0	0	0	0	0	0	0	0	0	0	0
Debaromyces	0	0	0	0	0	0	0	0	0	0	0.002878	0	0	0	0	0	0	0	0	0	0	0	0
Cyberlindnera	0	0	0	0	0	0	0	0	0	0	0.002878	0	0	0	0	0	0	0	0	0	0	0	0
Malassezia	0	0	0	0	0	0	0	0	0	0	0.002158	0	0	0	0	0	0	0	0	0	0	0	0

Supplemental Table 7: Relative abundances for the ITS milk (Part 1)

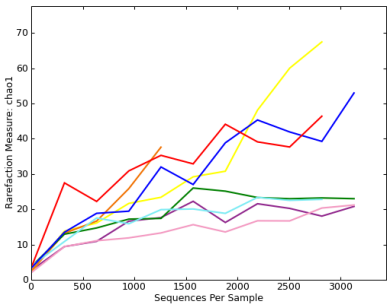
	BEL1	FR1	GER1	GER2	IR1	IR2	IR4	IR5	IR6	IR8	IR9	IR10	IT1	SP1	UK1	UK2	UK3	UK4	UK5	US1	US2	US3	US5
Genus																							
Heterobasidion	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00664	0	0	0	0	0	0	0	0
Neofabraea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.006496	0	0	0
Peziza	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.003511	0	0	0
Ganoderma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.005794	0	0	0
Mycena	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.005267	0	0	0
Dioszegia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.002107	0	0	0
Other	0.483478	0.026104	0.604458	0.451728	0.04717	0.007108	0.003528	0.002723	0.003027	0.012118	0.077938	0.106163	0.724552	0.015883	0.140278	0.613569	0.024046	0.243889	0.433924	0.311798	0.073	0.001842	0.957754
Species																							
Kazachstania barnetti	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kluyveromyces marxianus	0.501615	0.033244	0.338726	0	0.000529	0	0	0	0	0.000904	0.001679	0.001193	0.114724	0.001018	0.359203	0.379425	0.058665	0.039815	0.046881	0.044066	0.05753	0.010062	0.024285
Kazachstania unispora	0.012174	0.346497	0.056816	0.548272	0.948598	0.991965	0.399045	0.997277	0.988973	0.982094	0.068585	0.885288	0.157029	0.627774	0.467939	0.004425	0.360904	0.716296	0.519195	0.600597	0.869471	0.980726	0.008854
Naumovozyma castelli	0	0.593039	0	0	0	0	0.594107	0	0.005622	0.000904	0.818705	0	0	0.009978	0.017639	0	0.5483	0	0	0.018083	0	0	0.005818
Saccharomyces cerevisiae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Davidiella tassiana	0	0	0	0	0	0	0	0	0	0	0.008393	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium sp. *Vega 347*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pichia kudriavzevii	0	0	0	0	0	0	0	0	0	0	0	0	0	0.3427	0.003113	0	0.004768	0	0	0	0	0.001276	0.003289
Pichia occidentalis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pichia fermentans	0	0	0	0	0	0.000773	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dekkera anomala	0	0.001116	0	0	0	0	0.002283	0	0	0	0	0	0.00351	0.002647	0.00415	0	0.00228	0	0	0.001229	0	0	0
Dekkera bruxellensis	0.002733	0	0	0	0	0	0.001038	0	0	0	0	0	0	0	0	0.002581	0	0	0	0	0	0.004535	0
Zygosaccharomyces lentus	0	0	0	0	0.000441	0	0	0	0.002378	0.001266	0	0	0	0	0	0	0	0	0	0.001053	0	0.000709	0
Eurotium amstelodami	0	0	0	0	0.000705	0	0	0	0	0	0	0.00159	0	0	0	0	0	0	0	0	0	0	0
Wallemia sebi	0	0	0	0	0.002557	0	0	0	0	0	0	0.005765	0	0	0	0	0	0	0	0	0	0	0
Microdochium nivale	0	0	0	0	0	0	0	0	0	0.001809	0	0	0	0	0	0	0	0	0	0	0	0	0
Cryptococcus sp. Vega039	0	0	0	0	0	0	0	0	0	0.000904	0.001199	0	0	0	0	0	0	0	0	0	0	0	0
Teratosphaeria knoxdaviesii	0	0	0	0	0	0	0	0	0	0	0.001199	0	0	0	0	0	0	0	0	0	0	0	0
Cyberlindnera jadinii	0	0	0	0	0	0	0	0	0	0	0.002878	0	0	0	0	0	0	0	0	0	0	0	0
Malassezia pachydermatis	0	0	0	0	0	0	0	0	0	0	0.002158	0	0	0	0	0	0	0	0	0	0	0	0
Heterobasidion annosum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.001038	0	0	0	0	0	0	0	0
Paziza campestri s	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.003511	0	0	0
Ganoderma lucidum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.005794	0	0	0
Dioszegia hungarica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.002107	0	0	0
Other	0.483478	0.026104	0.604458	0.451728	0.04717	0.007262	0.003528	0.002723	0.003027	0.012118	0.095204	0.106163	0.724737	0.015883	0.146918	0.613569	0.025083	0.243889	0.433924	0.32356	0.073	0.002693	0.957754

Supplemental Table 7: Relative abundances for the ITS milk (Part 2)

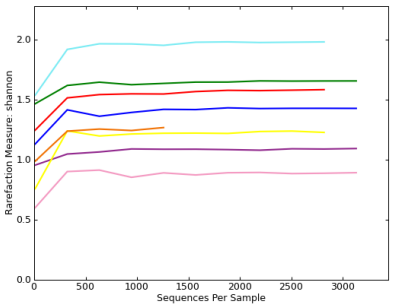
Appendix B: Supplemental information for Chapter 5

Sequence-based analysis of the microbial composition of water kefir from multiple sources

A) 16S

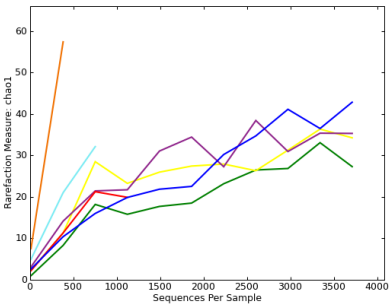


Chao1

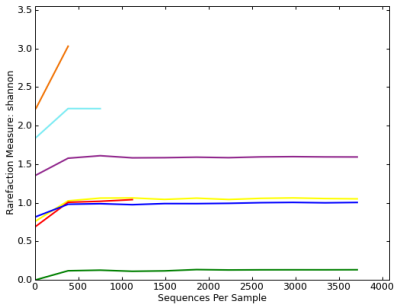


Shannon

B) ITS



Chao1



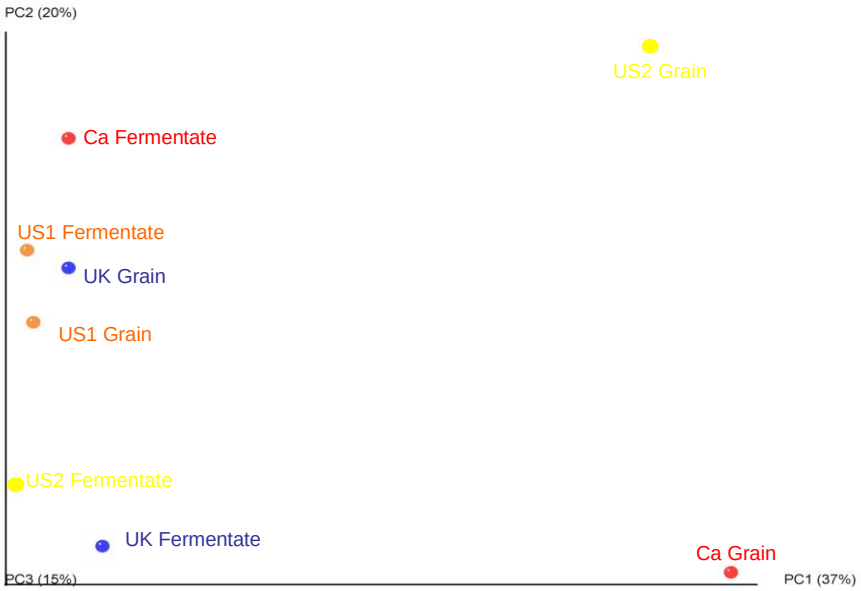
Shannon

Supplemental Figure 1

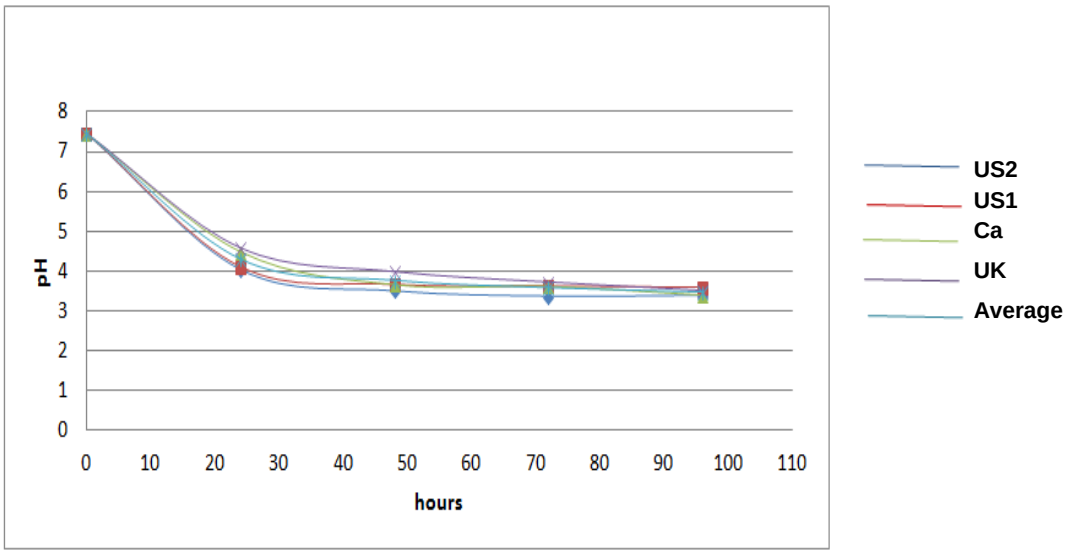
A) Rarefactions for the water kefir 16S samples

B) Rarefactions for the water kefir ITS samples.

Red = Ca fermentate, Blue = Ca grain, Orange = UK fermentate, Green = UK grain, Purple = US2 fermentate, Yellow = US2 grain, Light Blue = US1 fermentate



Supplemental Figure 2 Principle Coordinate Analysis (PCoA) plot based on unweighted UniFrac distance matrices, show distribution of the bacterial populations of water kefir grains and fermentates



Supplemental Figure 3 pH analysis of the different water kefirs over a 4-day period

	Chao1	Simpson	Shannon	Phylogenetic Diversity	Observed Species
Ca Fermentate	41.17	0.55	1.58	1.98	32
Ca Grain	48	0.47	1.43	2.4	26
UK Fermentate	23	0.58	1.65	1.49	20
UK Grain	43	0.41	1.26	1.31	15
US2 Fermentate	19.5	0.41	1.09	1.25	17
US2 Grain	89	0.42	1.23	2.19	23
US1 Fermentate	22	0.67	1.98	1.35	20
US1 Grain	25	0.25	0.89	1.3	15
Averages	38.83	0.47	1.39	1.66	21

Supplemental Table 1

Alpha Diversities for the Water Kefir 16S Population

	Fermentate				Grain			
	Ca	**UK**	**US1**	**US2**	**Ca**	**UK**	**US1**	**US2**
Total	3188	3186	3112	3633	3339	1408	3344	3062
Phylum								
Proteobacteria	0.5919	0.5964	0.5064	0.7479	0.7062	0.7571	0.8738	0.7495
Actinobacteria	0.005	0.0173	0	0.0014	0.0371	0.0781	0.0018	0.0251
Firmicutes	0.4015	0.3854	0.4923	0.2505	0.2558	0.1648	0.1241	0.224
Other	0.0016	0.0009	0.00129	0.0003	0.0009	0	0.0003	0.0013
Family								
Acetobacteraceae	0.0063	0.0298	0.0116	0.00712	0.0105	0	0.0039	0.0069
Sphingomonadaceae	0.5838	0.5659	0.4945	0.7407	0.6957	0.755	0.8696	0.7427
Bifidobacteriaceae	0.005	0.0172	0	0.0014	0.0362	0.0781	0.0018	0.0252
Lactobacillaceae	0.3896	0.3854	0.2346	0.2505	0.2549	0.1641	0.1196	0.2231
Leuconostocaceae	0.0116	0	0.2545	0	0	0	0.0045	0
Other	0.0038	0.0016	0.0048	0.0003	0.0027	0.0028	0.0006	0.0023
Genus								
Acetobacter	0.0035	0.0276	0.0103	0.0061	0	0	0	0
Zymomonas	0.5838	0.5659	0.4945	0.7407	0.6957	0.755	0.8696	0.7427
Lactobacillus	0.3883	0.3848	0.2339	0.2504	0.254	0.1641	0.1196	0.2231
Leuconostoc	0.0113	0	0.2526	0	0	0	0.0042	0
Gluconacetobacter	0	0	0	0	0.0093	0	0.0027	0.0052
Other	0.0132	0.0217	0.0087	0.0028	0.041	0.081	0.0039	0.0291

Supplemental Table 2

Relative abundances for the 16S population

	Fermentate 410 bp				Grain 410 bp			
	Ca	**UK**	**US1**	**US2**	**Ca**	**UK**	**US1**	**US2**
Total Reads	1549	192	1144	440	80	1186	23	51
Phylum								
Ascomycota	1527	192	1138	437	79	1186	23	51
Other	22	0	6	3	1	0	0	0
Family								
Saccharomycetaceae	313	192	354	336	63	1005	23	49
Saccharomycodaceae	1214	0	719	22	16	181	0	0
Debaryomycetaceae	0	0	9	17	0	0	0	0
Other	22	0	62	65	1	0	0	2
Genus								
Dekkera	212	185	0	139	42	260	0	25
Saccharomyces	99	0	279	181	0	629	0	0
Hanseniaspora	1214	0	719	22	16	181	0	0
Zygosaccharomyces	0	7	0	0	16	0	23	21
Meyerozyma	0	0	9	17	0	0	0	0
Torulaspora	0	0	69	15	0	37	0	0
Lachancea	0	0	0	0	0	74	0	0
Other	24	0	68	66	6	5	0	5
Species								
Dekkera anomala	202	0	0	139	10	205	0	25
Dekkera bruxellensis	10	185	0	0	32	55	0	0
Hanseniaspora valbyensis	1179	0	164	0	0	34	0	0
Hanseniaspora vineae	0	0	262	16	15	6	0	0
Zygosaccharomyces lentus	0	7	0	0	16	0	22	21
Lachancea fermentati	0	0	0	0	0	74	0	0
Saccharomyces cerevisiae	0	0	0	0	0	15	0	0
Other	158	0	718	285	7	797	1	5

Supplemental Table 3

Read numbers for the ITS 410 bp population

250 BP	Ca Grain	US2 Grain	UK Fermentate	US2 Fermentate
Total Reads	5540	6554	3524	8723
Phlyum				
Ascomycota	5540	6554	3524	8717
Family				
Saccharomycetaceae	5523	6553	3524	4497
Debaryomycetaceae	0	0	0	572
Genus				
Dekkera	5521	6549	3524	4493
Meyerozyma	0	0	0	572
Species				
Dekkera anomala	1008	6524	0	4489
Dekkera bruxellensis	4513	25	3523	0
Meyerozyma caribbica	0	0	0	23

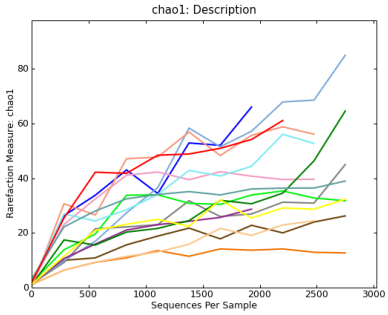
Supplemental Table 4

Read numbers for the ITS 250 bp population

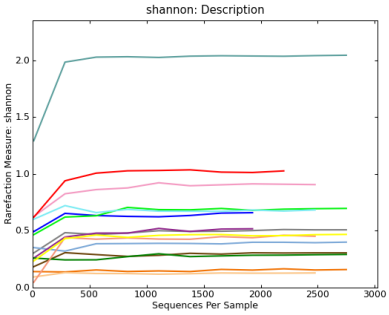
Appendix C: Supplemental information for Chapter 6

Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples

A) 16S

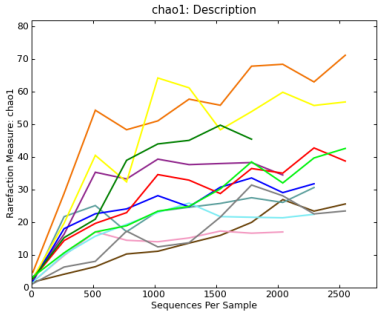


Chao1

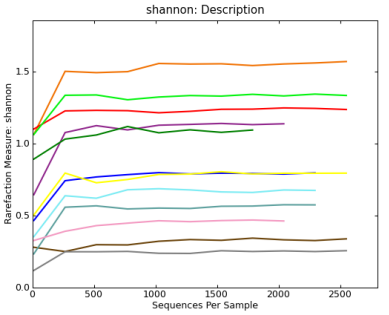


Shannon

B) ITS



Chao1



Shannon

Supplemental Figure 1

Chao1 and Shannon rarefaction curves for the bacterial (A) and fungal (B) portions of the kombucha microflora

16S	Average Length (bp)	Total Number of Reads	Reads Removed	Final Number
CA1 D3	408	2741	366	2348
CA2 D3	408	3615	596	3019
IRE D3	408	3008	379	2629
UK1 D3	408	3486	378	3108
US D3	408	3125	440	2685
CA1 D10	408	2693	495	2198
CA2 D10	408	2934	637	2297
IRE D10	408	3592	839	2753
UK1 D10	408	4263	524	3739
US D10	408	3487	598	2889
CA1 Pellicle	408	3612	440	3172
CA2 Pellicle	408	3541	352	3189
IRE Pellicle	408	4204	589	3615
UK1 Pellicle	408	3384	462	2922
US Pellicle	408	3184	396	2788

ITS	Average Length (bp)	Total Number of Reads	Reads Removed	Final Number
CA1 D3	~400	1673	100	1573
CA2 D3	~400	2147	116	2031
IRE D3	~400	2196	89	2107
UK1 D3	~400	605	13	592
US D3	~400	184	140	44
CA1 D10	~400	2468	118	2350
CA2 D10	~400	2301	120	2181
IRE D10	~400	2152	97	2055
UK1 D10	~400	1081	77	1004
US D10	~400	285	19	266
CA1 Pellicle	~400	6685	3564	3121
CA2 Pellicle	~400	2854	126	2728
IRE Pellicle	~400	2604	178	2426
UK1 Pellicle	~400	464	21	445
US Pellicle	~400	31	27	4
UK1 D3	~200	12607	302	12305
US D3	~200	1918	1780	138
UK D10	~200	31280	980	30300
US D10	~200	6439	188	6251
UK Pellicle	~200	3244	101	3143
US Pellicle	~200	678	217	461

Supplemental Table 1

Initial and post-trimming read numbers for 16S and ITS sequencing

	Chao1	Simpson	Shannon	Phylogenetic Diversity	Observed Species
CA1 Pellicle	12.75	0.031374628	0.158306172	1.1386	12
CA2 Pellicle	31.2	0.100538563	0.466323722	2.21427	24
IRE Pellicle	37	0.617060664	2.044298202	2.03325	34
UK1 Pellicle	32	0.165961616	0.696534652	1.72946	26
US1 Pellicle	27	0.024860042	0.13105602	0.78142	13
CA1 Day10	68.2	0.159216528	0.658246783	3.09973	34
CA2 Day10	25.66666667	0.131629396	0.517323461	1.43357	21
IRE Day10	39.09090909	0.236506545	0.90888707	2.43784	35
UK1 Day10	59	0.122930401	0.511389832	2.27834	26
US Day10	85	0.092146248	0.399950754	2.60514	28
CA1 Day3	65.2	0.244559744	1.027258337	3.51059	38
CA2 Day3	77	0.055905985	0.288686899	1.60541	22
IRE Day3	51.2	0.151218666	0.681989713	2.95005	33
UK1 Day3	29.5	0.061401348	0.304484265	1.32701	19
US1 Day3	56.375	0.091382697	0.452585328	3.6393	35
Averages	46.41217172	0.152446205	0.616488081	2.185598667	26.66666667

Supplemental Table 2

16S alpha diversities

	Chao1	Simpson	Shannon	Phylogenetic Diversity	Observed Species
CA1 Pellicle	73	0.410208718	1.561938465	9.5337	52
CA2 Pellicle	56.125	0.187998227	0.793375929	6.06477	37
IRE Pellicle	32.5	0.128781358	0.570382027	4.24074	22
CA1 Day3	42	0.524044423	1.246295154	2.24298	29
CA2 Day3	49	0.331495757	1.094396875	3.1122	28
IRE Day3	22.5	0.191558056	0.672859467	2.70784	19
CA1 Day10	32.2	0.21865987	0.793709461	3.47621	25
CA2 Day10	36.42857143	0.318411709	1.139318116	3.35537	30
IRE Day10	17	0.106323059	0.465173629	2.07843	15
Averages	40.08373016	0.26860902	0.926383236	4.090248889	28.55555556

Supplemental Table 3

ITS alpha diversities

Pellicle

Phylum	**Ca1**	**Ca2**	**Ire**	**UK**	**US**
Proteobacteria	0.99806	0.965049	0.600058	0.933216	0.998526
Firmicutes	0	0.032686	0.393599	0.065729	0
Actinobacteria	0	0	0.004325	0	0
Deinococcus-Thermus	0.001617	0	0	0	0
Other	0.000323	0.002265	0.002018	0.001054	0.001474
Family					
Acetobacteraceae	0.997736	0.957929	0.600058	0.932513	0.998526
Lactobacillaceae	0	0.017152	0.305652	0.046397	0
Streptococcaceae	0	0.012945	0.077566	0.016169	0
Thermaceae	0.001617	0	0	0	0
Propionibacteriaceae	0	0	0.003749	0	0
Enterococcaceae	0	0	0.007209	0.002109	0
Other	0.000647	0.011974	0.005767	0.002812	0.001474
Genus					
Acetobacter	0	0	0.019319	0.002812	0
Gluconacetobacter	0.997736	0.957282	0.580161	0.929701	0.998158
Lactobacillus	0	0.017152	0.305652	0.046397	0
Lactococcus	0	0.012945	0.077566	0.016169	0
Enterococcus	0	0	0.00692	0.002109	0
Propionibacterium	0	0	0.003749	0	0
Other	0.002264	0.012621	0.006632	0.002812	0.001842

Day 3

Phylum	**Ca1**	**Ca2**	**Ire**	**UK**	**US**
Proteobacteria	0.928695	0.982958	0.938383	0.983411	0.9596
Firmicutes	0.063526	0.016701	0.056122	0.015594	0.034244
Actinobacteria	0.003457	0	0.002747	0	0
Deinococcus-Thermus	0.002161	0	0.001962	0	0
Bacteroidetes	0	0	0	0	0.003848
Other	0.002161	0.000341	0.000785	0.000995	0.002309
Family					
Acetobacteraceae	0.883319	0.97955	0.935636	0.982415	0.95883
Lactobacillaceae	0.039758	0.011247	0.035714	0.011944	0.017699
Streptococcaceae	0.015557	0.005112	0.017661	0.00365	0
Leuconostocaceae	0.005186	0	0	0	0
Bifidobacteriaceae	0.003025	0	0	0	0
Thermaceae	0.002161	0	0.001962	0	0
Propionibacteriaceae	0	0	0.001962	0	0
Ruminococcaceae	0	0	0	0	0.002693
Erysipelotrichaceae	0	0	0	0	0.00885
Lachnospiraceae	0	0	0	0	0.004617
Other	0.050994	0.00409	0.007064	0.001991	0.007311

Supplemental Table 4 (Part 1)

Relative abundances of the 16S bacterial populations at phylum, family and genus level.

Day 3

Genus	Ca1	Ca2	Ire	UK	US
Acetobacter	0.008643	0	0.004317	0	0
Gluconacetobacter	0.869058	0.977846	0.930926	0.98142	0.957291
Lactobacillus	0.039326	0.011247	0.035714	0.011944	0.017699
Lactococcus	0.015557	0.005112	0.017661	0.00365	0
Leuconostoc	0.005186	0	0	0	0
Bifidobacterium	0.002593	0	0	0	0
Thermus	0.002161	0	0.001962	0	0
Allobaculum	0	0	0	0	0.00885
Ruminococcaceae Incertae Sedis	0	0	0	0	0.001924
Propionibacterium	0	0	0.001962	0	0
Other	0.057476	0.005794	0.007457	0.002986	0.014236

Day 10

Phylum	Ca1	Ca2	Ire	UK	US
Proteobacteria	0.925422	0.933393	0.87848	0.944214	0.960246
Firmicutes	0.065197	0.064356	0.105342	0.017122	0.037947
Actinobacteria	0	0	0	0	0
Deinococcus-Thermus	0.007974	0	0.016178	0.037559	0
Bacteroidetes	0	0	0	0	0
Other	0.001407	0.00225	0	0.001105	0.001807
Family					
Acetobacteraceae	0.923077	0.932493	0.878104	0.94311	0.958439
Lactobacillaceae	0.059568	0.061656	0.095937	0.014361	0.034695
Streptococcaceae	0	0.00225	0.002257	0	0.001807
Thermaceae	0.006567	0	0.016178	0.037283	0
Bacillaceae	0	0	0	0.001381	0
Other	0.010788	0.0036	0.007524	0.003866	0.00506
Genus					
Acetobacter	0	0	0.001881	0	0
Gluconacetobacter	0.92167	0.931593	0.876223	0.942557	0.957355
Lactobacillus	0.059568	0.061656	0.095937	0.014361	0.034695
Lactococcus	0	0.00225	0.001881	0	0.001807
Thermus	0.006567	0	0.016178	0.037283	0
Other	0.012195	0.0045	0.007901	0.0058	0.006144

Supplemental Table 4 (Part 2)

Relative abundances of the 16S bacterial populations at phylum, family and genus level.

Pellicle Phylum	Ca1	Ca2	Ire
Ascomycota	0.947164	0.943667	0.997093
Basidiomycota	0.008428	0.055596	0
Other	0.044408	0.000736	0.002907
Family			
Saccharomycetaceae	0.824311	0.942194	0.990864
Davidiellaceae	0.001621	0	0
Clavicipitaceae	0.001621	0	0
Pichiaceae	0.115397	0	0.006229
Wallemiaceae	0.005835	0	0
Leucosporidiaceae	0	0.055596	0
Debaryomycetaceae	0	0	0
Saccharomycodaceae	0	0	0
Other	0.051216	0.002209	0.002907
Genus			
Dekkera	0.010697	0.00405	0
Zygosaccharomyces	0.789627	0.932622	0.953904
Davidiella	0.001621	0	0
Pichia	0.115397	0	0.006229
Wallemia	0.005835	0	0
Lachancea	0	0.002946	0
Leucosporidiella	0	0.055596	0
Kazachstania	0	0	0.003322
Kluyveromyces	0	0	0.002492
Naumovozyma	0	0	0.005399
Meyerozyma	0	0	0
Saccharomyces	0	0	0
Hanseniaspora	0	0	0
Other	0.076823	0.004786	0.028654
Species			
Dekkera bruxellensis	0	0.001841	0
Dekkera anomola	0	0.002209	0
Zygosaccharomyces bisporus	0	0.060015	0
Zygosaccharomyces lentus	0.788655	0.872607	0.953904
Pichia kudriavzevii	0.114749	0	0.006229
Davidiella tassiana	0.001621	0	0
Wallemia sebi	0.005835	0	0
Lachancea fermentati	0	0.002946	0
Leucosporidiella fragaria	0	0.055596	0
Kazachstania unispora	0	0	0.003322
Kluyveromyces marxianus	0	0	0.002492
Naumovozyma castellii	0	0	0.005399
Hanseniaspora vineae	0	0	0
Other	0.089141	0.004786	0.028654

Supplemental Table 5 (Part 1)

Relative abundances of the ITS fungal populations at phylum, family, genus and species level.

Day 3

Phylum	Ca1	Ca2	Ire
Ascomycota	1	1	0.999047
Other	0	0	0.000953
Family			
Saccharomycetaceae	1	1	0.998571
Other	0	0	0.001429
Genus			
Dekkera	0.0057	0	0
Zygosaccharomyces	0.9943	0.998026	0.995712
Kazachstania	0	0	0
Other	0	0.001974	0.004288
Species			
Dekkera bruxellensis	0.0057	0	0
Dekkera anomola	0	0	0
Zygosaccharomyces bisporus	0.015833	0.173741	0
Zygosaccharomyces lentus	0.978467	0.824284	0.995712
Kazachstania unispora	0	0	0
Other	0	0.001974	0.004288

Day 10

Phylum	Ca1	Ca2	Ire
Ascomycota	1	1	1
Other	0	0	0
Family			
Saccharomycetaceae	0.999573	0.99954	1
Other	0.000427	0.00046	0
Genus			
Dekkera	0.004268	0.007363	0.008264
Zygosaccharomyces	0.991891	0.991256	0.986874
Kazachstania	0	0	0
Other	0.003841	0.001381	0.004861
Species			
Dekkera bruxellensis	0.002988	0.006903	0.005348
Dekkera anomola	0	0	0.002917
Zygosaccharomyces bisporus	0.020487	0.147262	0
Zygosaccharomyces lentus	0.971404	0.843994	0.986874
Kazachstania unispora	0	0	0
Other	0.005122	0.001841	0.004861

Supplemental Table 5 (Part 2)

Relative abundances of the ITS fungal populations at phylum, family, genus and species level.

	Pellicle 400bps	Day 3 400bps	Day 10 400bps	Pellicle 250bps	Day 3 250bps	Day10 250bps
Reads	444	12888	1003	3142	12298	30292
Phlyum						
Ascomycota	436	12882	1002	3137	12292	30279
Basidiomycota	6	0	0	0	0	0
Other	2	6	1	5	6	13
Family						
Saccharomycetaceae	400	12881	1000	3100	12291	30279
Debaryomycetaceae	5	0	0	16	0	0
Saccharomycodaceae	6	0	0	0	0	0
Leucosporidiaceae	6	0	0	0	0	0
Other	27	7	3	26	7	13
Genus						
Dekkera	276	12379	143	3097	12282	30270
Zygosaccharomyces	76	502	857	0	9	0
Leucosporidiella	6	0	0	0	0	0
Kazachstania	0	0	0	0	0	5
Meyerozyma	5	0	0	16	0	0
Saccharomyces	46	0	0	0	0	0
Hanseniaspora	6	0	0	0	0	0
Other	29	7	3	29	7	17
Species						
Dekkera bruxellensis	203	12379	143	2375	12282	30260
Dekkera anomola	73	0	0	722	0	10
Zygosaccharomyces lentus	76	502	857	0	9	0
Leucosporidiella fragaria	6	0	0	0	0	
Kazachstania unispora	0	0	0	0	0	5
Hanseniaspora vineae	5	0	0	0	0	0
Other	81	7	3	45	7	17

Supplemental Table 6

ITS read numbers for UK kombucha, from two sequencing pools of approximate sizes 400 bp and 250 bp

DISCUSSION

Bacteriocins are the focus of ever-increasing levels of interest. Currently, the commercial application of bacteriocins is limited to the treatment of skin infections, in food preservation and the use of bacteriocin producers in oral lozenges, but it is likely that additional clinical and vetinary applications will occur in the near future. The emergence of antibiotic-resistant bacteria combined with the poor rate of novel antibiotic discovery, represent enormous challenges for modern medicine, and bacteriocins have the potential to provide solutions to these issues. In a literature review for this thesis (chapter 1), strategies that can be used to identify novel antimicrobials are discussed. Of these strategies, we applied *in-silico* mining and high-throughput robotics with a view to identifying novel antimicrobials.

Genome mining for ribosomally synthesised products is a very useful technique (Velasquez and van der Donk, 2011), and in the past has been applied to screen for bacteriocin producers from Gram negative bacteria and from wine (Knoll et al., 2008, Dirix et al., 2004). We screened the fully sequenced genomes within the BLAST database for novel bacteriocins using the biosynthetic machinery enzymes from the bacteriocin nisin as driver sequences. By applying an *in-silico* screen to search for novel homologs, we revealed the presence of lantibiotic genes within multiple genomes of bacteria from diverse ecological niches, such as deep sea vents, soil and the gastrointestinal tract. This approach has been shown to translate to the identification of functional antimicrobial peptides by laboratory proof-of-concept investigations in the case of bacteriocins lichenicidin (Begley et al., 2009), Bsa (Daly et al., 2010) and venezuelin (Goto et al., 2010).

While the identification of gene clusters using *in-silico* approaches does not necessarily ensure that the strain in question is a bacteriocin producer, it is revealing that such antimicrobial genes are present in many different bacteria, reflecting a combination of the origin of these clusters as well as more recent gene transfers. This strategy has since been applied for lantibiotic LanM biosynthetic enzymes (O'Sullivan et al., 2011), sactibiotic radical SAM protein determinants (Murphy et al., 2011) and bacteriocins in cyanobacteria (Wang et al., 2011).

The development of strategies for *in-silico* bacteriocin mining reflects the vast number of ribosomally synthesised antimicrobial genes in sequence databases. This is increasingly relevant with the rise in number of genome sequences, as evidenced by the fact that the number available at the time of our study, which was approximately 1178, has since increased over three fold. A specific online program, BAGEL (BActeriocin GEnome mining tooL) (de Jong et al., 2006), was designed for this purpose. When the studies in chapter 3 were carried out, BAGEL was in its first iteration. It has since undergone further revision, and is currently at BAGEL3 (van Heel et al., 2013). BAGEL recognises various antimicrobial operon motifs and has been utilised in several studies since. Additionally, the development of databases, such as Bactibase (Hammami et al., 2010), is reflective of the growing need to accurately accrue and organise bacteriocin data.

The global functional food and drink market is expected to be worth $29.8 billion by 2014. A major factor in the success of fermented functional beverages are their bio-active components, i.e. microbes or bioactives produced

by microbes, which are usually supplied in the form of dairy beverages. Due to this success, there is a keen interest to access this market through the development of new beverages with functional properties. To this end, it is notable that there are many traditional fermented beverages which have been used by different societies throughout history, and many are believed to aid in the treatment of gastrointestinal ailments. These could be harvested for health-promoting bacteria and used to derive commercial functional beverages for the modern health-conscious market. Additionally, such beverages have the potential to serve as cheap, effective means of delivering nutrition to deprived populations, and as a vehicle for probiotic delivery.

The majority of these beverages have not been extensively studied, with even fewer studies focusing on supporting health claims. However, the first step toward the commercial production of these beverages is to understand the microorganisms responsible for the fermentation, which can also shed light on the delicate symbiosis between the bacteria and yeast present. We were particularly interested in revealing the differences between the microbial populations within the solid inoculating materials and the resulting fermentates, the change in these populations over the course of the fermentation and the differences between beverages themselves sourced from geographically distinct regions.

To achieve this goal, the aim was to use a culture-independent technology, i.e. high-throughput sequencing, to investigate the microbial populations. This technology, probably best known for its use by the human genome project, has since been applied to a variety of environments including

aqueous locations, forest soils and, frequently, the gastrointestinal tract, and has revolutionised many fields of research. With respect to fermented foods, the application of high-throughput sequencing has already been used to assess the populations of many fermented foods from kimchi to cheese (Roh et al., 2010, Quigley et al., 2012), and has revealed a number of valuable insights. The advantages of such technologies over traditional culture-based methodologies are several fold, including the ability to detect populations which are either unculturable, or present in such low numbers that they avoid detection in culture-based methods, and also avoids bias due to culturing in the absence of appropriate nutrients or at specific temperatures. Additionally, this method is high-throughput, offering relative population abundance, unlike other molecular techniques such as DGGE which can also only detect 1-2% of the microbial population (Muyzer et al., 1993). The merit of our sequencing-based studies was enhanced by the inclusion of fungal-specific primers to investigate the fungal component, as well as the bacterial component, of these populations. This development was particularly important when studying these symbiotic microorganisms, and ultimately provided a comprehensive view of the microbial communities present.

For the bacterial component of these populations, the degenerate PCR primers we used were designed to amplify the variable V4-V5 region of the 16S rRNA gene (Claesson et al., 2012), generating amplicons of of 408 bp. These amplicon sizes are relatively longer than those used in many previous microbial diversity studies and take full advantage of the longer reads provided by GS-FLX Titanium technologies. While this facilitated more confident assignments, it was

still not possible to assign reads beyond the genus level due to the high level of sequence similarity in the 16S rDNA gene. The limiting factor with respect to accurately assigning at the species level relates to technology rather than an issue with primer design, whereby current high-throughput sequencing systems are unable to generate the longer reads that could lead to greater taxonomic resolution. Currently, 16S Sanger-sequencing based analysis of specific colonies is still of some interest, if only to accurately identify species.

The issue of accurately assigning at species level was not a problem for sequencing of the internal transcribed spacer regions of fungal species, which is sufficiently heterogenous to assign sequences at the species level. The metagenomic sequencing of yeast/fungal populations is not as routine as for bacterial populations and only recently has a consensus emerged that the ITS region should be used. Even still, it is argued that several approaches are required to accurately assess populations. While this might be true, this is unlikely to be as relevant for the relatively low diversity, and predominantly yeast populations, that have been the focus of our studies. The emergence of a larger database of ITS sequences will prove particularly useful for future studies.

The use of culture-independent techniques to assess yeast environments is particularly advantageous from a taxonomic viewpoint. Many yeast exist in two forms, a sexual (teleomorphic) and asexual (anamorphic) state, and based on phenotypic classification have been classed as separate microorganisms (e.g. *Dekkera*/*Bruxellensis*, *Candida*/*Pichia*, *Kloeckera*/*Hanseniaspora* etc), despite having the same genetic content. It has been proposed that by this year (2013), studies refer to species in their teleomorphic state only, unless there are

extenuating circumstances. With the availability of increasing genetic information, yeast are regularly reclassified, further adding to the confusion. For example, at first glance, the population profile of fungi in kefir, as shown in chapter 4, are quite different to those of previous studies. There is a noticeable lack of *Candida* and *Saccharomyces*, conventionally associated with kefir, but the explanation for this pattern is that species of these genera have been reclassified to *Kluyveromyces* and *Kazachstania*, respectively. Many yeast are considered unculturable and reliable culture-independent identification is likely to reveal many new species in the future. In our studies, there are a number of reads that, while belonging to the phylum *Ascomycota*, could not be assigned to a genus within the molecular database. This is most likely due to the fact that there remain so many microorganisms to be identified and corresponding ITS sequences deposited in such databases. In fact, it has even recently been suggested that the volume of yeast set to be uncovered by molecular inspection might be best served by numerical assignments rather than Latin nomenclature (Money, 2013).

The analysis of kefir was devised as a follow-up study to a previous publication by Dobson *et al*, which used 454 sequencing to analyse the population of a single kefir grain and milk (Dobson et al., 2011). Our aim was to expand on these results, and to use the same technology to assess both the grains and milks of kefirs sourced from distinct regions around the world, and was the largest of our three microbial diveristy studies. Our results confirm the findings of Dobson *et al*, by showing that kefir grains are most often dominated by *Lactobacillus*, while the most abundant population in the milks were

Lactococcus. It was also shown that the proportions of *Acetobacter* vary from kefir to kefir while *Leuconostoc* increased from all grains to milks. The abundance of both these genera in the milk are likely to effect the composition through acetic acid and slime production respectively. With regards fungal composition, our analysis revealed the most prominent genera to be *Kazachstania*, *Naumovozyma* and *Kluyveromyces*. The identification of *Naumovozyma* is particularly interesting, in that it has not been revealed as a component of kefir previously. Beta analyses revealed that the different kefirs did not cluster, and were dissimilar from one another regardless of geography.

Water kefir is a beverage that has become increasingly popular beverage with putative functional properties, that is available from artisanal health stores in the western world. Currently, it is most popularly advertised as a non-dairy alternative to milk-based kefir, offering similar health-promoting microbes. However, as our study shows, this is not always the case. A 16S high-throughput sequencing study based on the microbial populations of water kefir released shortly before our publication (Gulitz et al., 2013), showed that the populations are dominated by *Lactobacillus*, with a significant number of bifidobacteria. The single biggest difference between our study and theirs, and, indeed, all other existing water kefir studies, was the dominant presence of *Zymomonas* in each of the four water kefirs analysed. This was a surprising find, especially given its consistent presence in each sample, and in similar proportions in the grains and fermentates. Although the sucrose/mineral-water solution had been sterilized before inoculation, the addition of a single fig to each culture may have provided a means through which this microorganism became part of the water

kefir population. The purpose of the adding the fig was to simulate a traditional fermentation, whereby dried fruit (most popularly a fig) are included to provide additional nutrients and flavour. The study by Gulitz *et al*. instead used a sterile fig solution which is less reflective of traditional approaches. This observation raises several questions regarding the stability of the the microbial populations traditionally associated with the water kefir, the proportion of dextran-producing bacteria required for grain formation and the significance of the apparently transient nature of the microbial population with respect to safety. Further research is required to understand the nature of the relationship between the individual components of water kefir microbes, the effects different substrates exert on this microbial population and the effects that individual taxa and substrates have on the physiochemical attributes of the beverage. Ultimately, however, although water kefir has yet to be commercialised on a significant scale, further studies with a view to its use as a vehicle for health-promoting microorganisms, merit attention.

Kombucha population analysis was useful in that it underscored the benefits of using molecular techniques for accurate taxonomic classification. More specifically, many species of *Acetobacter* have been reclassified as *Gluconacetobacter*, which was identified as the dominant genus in kombucha. *Gluconacetobacter* may also have been overlooked previously due to the difficulties in the phenotypic differentiation of acetic acid bacteria. It should also be noted that the bacterial component of kombucha has not been the focus of recent studies, possibly as a consequence of the perception that these populations are composed of acetic acid bacteria only. By investigating this

component, we found that *Lactobacillus* constituted a greater proportion of the consortium than was previously appreciated. It is suspected that this is due to the difficulty in culturing the strains present, as no lactobacilli were isolated from kombucha during our screening studies. The fact that up to one fifth of the bacterial component of kombucha can be LAB, and that quantities of bifidobacteria can also be present, shows promise for the inclusion of health-promoting starter cultures in the future.

Intruigingly, in certain water kefir and kombucha samples, DNA amplification of the ITS region generated two bands of approximately 250 bps and 410 bps in size. Prior to DNA sequencing, it was not apparent which taxa were represented by the different bands, and there are very few references in the literature to this phenomenon. As shorter reads are preferentially sequenced, pooling of the different-sized amplicons was not possible. It was therefore necessary to sequence both bands seperately. Almost all reads from the 250 bp pool were assigned to *Dekkera*, with only a small number assigned to *Meyerozyma*.

The field of bioinformatics is constantly evolving. As an example, MEGAN, which we used for DNA sequence assignments, was updated with a new version of the SILVA ribosomal database (version 106) during the course of our studies. Prior to this, most sequences from the water kefir samples could only be assigned to the level of family, namely, *Sphingomonadales*, with few reads assigned at the genus level. However, use of the new version allowed us to assign reads from this family to the genus *Zymomonas*.

The information gained from these analyses highlights how significantly the microbial content of these beverages can vary. This should prove valuable for the design of starter cultures to facilitate large scale commercial production of these beverages and in linking specific populations with specific physiochemical attributes.

With the advancement of technology and bioinformatics, these 16S analyses should become more affordable and commonplace. In the future, more integrated systems, with uniform methods for DNA extraction and amplification, and improvements in taxonomic databases and data analysis, will emerge. We are still at the beginning of this emerging field, and it is likely that these technologies will play a role in examining the microbiology of food for quite some time to come (Ercolini, 2013).

The final chapter of this thesis focused on both microbes sourced from fermented beverages and bacteriocins. Bacteriocins have many applications in the food industry, where they can be used as natural alternatives to chemical preservatives, can aid in the ripening of cheese and have also been associated with probiotic function (Dobson et al., 2012, Cleveland et al., 2001). Bacteriocin producers sourced from food environments are more likely to be of value with respect to subsequent food applications.

It was our intention to screen the fermented beverages kefir, water kefir and kombucha for the presence of antimicrobial bacterial producers, as fermented foods have long proven to be a good source of bacteriocin-producers. A large bank of isolates was created using robotics. Unfortunately, no novel bacteriocins were discovered, which was surprising considering the

geographical diversity and number of samples included in the screen. It could be that antimicrobials are only produced by these isolates under specific circumstances, and that the appropriate temperature, pH, nutrients or indicator were not employed.

However, the accidental discovery of a yeast strain producing a proteinaceous antimicrobial was interesting. Purification of the antimicrobial proved difficult, but was determined to be >30 kDa, and possibly contains more than one component. Yeast are known to produce large antimicrobial proteins, which are analogous to bacteriocins, but these are typically active against other yeast. Despite screening against some species of yeast, no activity was seen, but it could be that a larger range of indicators is required. To determine the exact nature of the protein, further analysis is required, which time did not permit. At the very least, the screen suggests that yeast from these environments are also a potential source of novel antimicrobials, and should be the focus of further screens in the future.

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